1-1-1991

Nuclear polyhedrosis virus of the gypsy moth (Lymantria dispar L.) : environmental and ecological factors influencing transmission and host susceptibility.

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NUCLEAR POLYHEDROSIS VIRUS OF THE GYPSY MOTH (*LYMANTRIA DISPAR* L.): ENVIRONMENTAL AND ECOLOGICAL FACTORS INFLUENCING TRANSMISSION AND HOST SUSCEPTIBILITY

A Dissertation Presented

by

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Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 1991

Department of Entomology

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ACKNOWLEDGMENTS

I have many people to thank for their help in completing this project. First of all my advisor, Joe Elkinton, generously provided technical and financial support throughout my program and supplied encouragement and motivation as needed. The other members of my committee were also extremely helpful. John Burand patiently 'persisted' through many hours with me in the lab as I learned the difference between microliters and molar solutions at the expense of his centrifuge. John Podgwaite, of the US Forest Service, provided lab space, technical help, and NPV, in addition to his expert advice and realistic counsel. Leonard Norkin first enlightened me to the fascinating realm of viruses and provided much insight for my research.

I thank the director, Chuck Schwalbe, and all the staff at the USDA Otis Methods Development Center for providing a great deal of assistance, laboratory space, insects, and the defoliation festival. I am especially endebted to Rich Hansen, Jesse Baker, John Tanner, Vic Mastro, Becky Upton, Gary Bernon, Juliet Tang, and Alida Pelligrini-Toole. Kathleen Shields of the US Forest Service and Martin Shapiro of the USDA-ARS frequently gave me invaluable suggestions and carefully reviewed earlier versions of several chapters. I would like to thank Les Perry and the staff at Shawme-Crowell State Forest, Crane Wildlife Refuge, and the Massachusetts Air National Guard for making study sites

available. Ralph Webb of the USDA-ARS and Kathleen Tatman, Steve Tilley, Deborah Godfrey, and Elizabeth Kinlock, of the Maryland Department of Agriculture greatly helped me in establishing study sites and collecting insects in Maryland.

At the University of Massachusetts, Steve Woods, Juli Gould, Sandy Liebhold, Bob Childs, Carol Ferguson and Steve Keating helped me on countless occasions with rainy field days, late nights, ELISA failures, and statistical nightmares. Jeff Boettner and Cynthia Whipple Boettner helped immensely with everything from graphics to cold-weather egg mass collecting. Carol Ferguson kept me sane and aerobically fit, while Mary, Holly, Eying Chen and Debbie kept things fun. Don Weber and Martin Aluja-Schunemann were great study partners. Ann Hazzard and Ruth Hazzard were conscious and compassionate company. I am thankful to the Green's for a fun, warm home, complete with a cat and video games. Many students energetically assisted in the laboratory and showed valor in the face of Lyme Disease and yellow jackets in the field. I am especially grateful to Jon Foulkes, Bob DeManche, Bob Sherman, Debbie Moreau, Valerie Tingley, Nedil Aldorondo, Sandra Lary, John Priest, Karin Fischer, Ellen Clark, Margi Coggins, Iris Garcia, Liz Harrahy, Alexia Bonser, Christina Kish, and Dee Gomes.

Leon Tsomides helped me with everything and was my best friend through it all.

ABSTRACT

NUCLEAR POLYHEDROSIS VIRUS OF THE GYPSY MOTH (*LYMANTRIA DISPAR* L.): ENVIRONMENTAL AND ECOLOGICAL FACTORS INFLUENCING TRANSMISSION AND HOST SUSCEPTIBILITY

FEBRUARY 1991

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The gypsy moth, *Lymantria dispar* L., is one of the most important forest pests in the United States. Insecticides are frequently used to suppress outbreaks, however, untreated outbreaks generally collapse due to disease epizootics caused by a nuclear polyhedrosis virus (NPV). Knowledge of NPV epizootiology could aid in the use of NPV as a biological control agent.

In this study, the mechanisms by which the NPV is transmitted across host generations, factors affecting host susceptibility to the pathogen, and the effects of NPV on the behavior and distribution of infected larvae in natural populations were investigated. A series of experiments were conducted in the field to determine whether maternal or environmental mechanisms are responsible for transgenerational transmission of the pathogen. Larvae

hatching from egg masses oviposited in NPV-contaminated environments became infected, whereas, progeny of NPV-fed parents did not, indicating that environmental contamination of egg masses, rather than become maternal transmission, is the principle means of transmission across generations. Further tests showed that egg masses acquire inoculum from the substrate during oviposition, rather than through rain-caused leaching.

The pathogenesis of the virus in gypsy moth larvae, pupae, and adults was followed using nucleic acid-hybridization techniques. Virus was not detected in adult moths suggesting that vertical transmission of NPV from infected moths to progeny may not be possible in the gypsy moth.

Susceptibility to disease may also be affected by disease prevalence. In laboratory assays, larvae from populations with more heavily NPV-contaminated eggs were more resistant to NPV than were populations with little inoculum present. These results suggest that exposure to NPV may favor selection for resistance to the pathogen.

It was also demonstrated that NPV infection alters the behavior of larvae, resulting in the tendency of infected larvae to die in the upper crowns of trees. Thus, samples of larvae collected from the upper canopy contain a higher proportion of infected larvae compared with those collected from under burlap bands wrapped around tree trunks. Therefore, NPV prevalence is more precisely estimated by

rearing larvae collected by a stratified-random sampling design.

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INTRODUCTION

Since its accidental introduction from Europe in the late 1860's, the gypsy moth, Lymantria dispar L., has become one of the most important forest insect pests in the United States (Liebhold et al. 1989). In the Northeast, where it is well established, control efforts are aimed at managing this pest and its damage, however, in other areas, notably the Pacific Coast states, eradication programs have been mounted to prevent its establishment (Dreistadt and Dahlsten 1989). In addition to economic losses associated with tree deformity, growth loss, and mortality caused by gypsy moth defoliation, indirect effects such as increased fire hazard, watershed and wildlife habitat damage, water contamination, and deleterious effects on human health and esthetics are also of primary concern (White and Schneeberger 1981).

In the absence of control efforts, gypsy moth outbreaks usually collapse due to epizootics, or widespread disease, caused by a nuclear polyhedrosis virus (NPV) (Bess et al. 1947, Doane 1970, Woods and Elkinton 1987). Because NPV appears to play such a major role in gypsy moth population dynamics, knowledge of the causes and patterns of expression of this disease is potentially very valuable for gypsy moth pest management.

Unfortunately there are still many gaps in our understanding of NPV epizootiology. Although this virus has

been formulated and registered for use as a microbial insecticide (Gypchek), the results of field trials have often been disappointing (Lewis and Yendol 1981, Podgwaite 1985, Yendol et al. 1977). Insight into the processes by which naturally occurring NPV acts to regulate gypsy moth populations might be used to improve efficacy of microbial insecticide applications or to enhance the occurrence of natural epizootics.

The gypsy moth NPV, like other members of the Baculovirus group, is an enveloped virus with a double-stranded, covalently-closed DNA genome (Evans and Entwistle 1987). In the nuclei of infected host cells, the virus replicates and directs the production of the polyhdedral inclusion body (PIB), a crystalline protein matrix, which becomes embedded with enveloped bundles of virions (Granados and Williams 1986). The occluded virions are released in abundance from the liquified cadavers of infected larvae and can survive outside of the host, protected from environmental degradation by the PIB, for many years (David and Gardiner 1967, Thompson 1978). Larvae become infected through ingestion of PIBs (Evans 1986, Evans and Harrap 1982). Infection is initiated when alkaline juices in the insect gut digest the PIBs, releasing virions which enter the host tissues via the midgut (Granados and Williams, 1986).

Recent studies have elucidated some of the ecological factors affecting the spread of NPV in gypsy moth

populations. For example, epizootics appear to develop through host density-dependent processes (Campbell 1963, Doane 1969, 1970, 1975, 1976a, 1976b, Woods and Elkinton 1987). First instars, which become infected upon hatching from contaminated egg masses (Doane 1969, 1975, Leonard and Doane 1966), or by ingestion of PIBs acquired by walking on contaminated substrates (Weseloh and Andreadis 1986, Woods et al. 1989), die and serve as primary inoculum for later instars, resulting in a 'second wave' of mortality (Doane 1969, 1976a, 1976b, Higashiura and Kamijo 1978, Woods and Elkinton 1987, Woods et al. 1990). Regulation of invertebrate hosts by density-dependent cycling of virulent pathogens, such as NPV, is predicted by mathematical models developed by Anderson and May (1980, 1981).

An important source of NPV in gypsy moth populations is the inoculum, sometimes referred to as the 'virus load', associated with egg masses, expressed as infection and mortality among first instars (Bergold 1942, 1943, Doane 1969, 1970, 1975, Leonard and Doane 1966). Doane (1976a) reported NPV-caused mortality exceeding 90% among first instars hatching from single egg masses in dense populations. Thus, the egg mass virus load can be an important source of inoculum for the 'first wave' of mortality among early larval stadia. Because the gypsy moth overwinters in the egg stage, this is also important as a means of maintaining NPV in a population from one year to

the next. However, the processes by which eggs become infected or contaminated with NPV have never been examined.

Transgenerational Transmission of NPV in Gypsy Moth Populations

In theory, virus could be transmitted to eggs by vertical (maternal) transmission, or by contamination from the environment. It has been suggested in much of the early literature that viruses are readily transmitted within eggs (transovarial transmission) laid by infected adults (Longworth and Cunningham 1968, Swaine 1966). However, virtually all NPV associated with gypsy moth eggs can be inactivated by surface disinfection (Doane 1969), therefore, it seems unlikely that much, if any, transovarial transmission occurs in this insect.

Doane (1976a) and Podgwaite et al. (1981) suggested that infected moths deposit virus onto the egg surface (transovum) at the time of oviposition. Shapiro and Robertson (1987) reported low infection rates occurring among the offspring of sublethally dosed parents, lending support to the notion of maternal transmission, although the mechanism by which progeny became infected was not examined. However, in a similar study, Shields (1984) found no evidence of transmission, nor any deleterious effects among progeny of sublethally dosed gypsy moths. Evans (1986)

argued that transmission by infected adults to offspring may be limited to another group of insects, the sawflies, which can harbor gut infections in the adult stage. Vertical transmission among the Lepidoptera may occur solely through mechanical transfer of inoculum from externally contaminated, rather than internally infected adults to eggs. Such a mechanism could also be invoked to explain the results presented by Shapiro and Robertson (1987).

A prerequisite for maternal transmission by infected adults to offspring is that sublethal infections acquired in the larval stage are maintained through the pupal and adult stages. Although it is well known that a certain percentage of insects that ingest NPV do not succumb to disease (Bakhvalov et al. 1982, Shields 1984), there is some disagreement as to whether these survivors harbor persistent infections, or whether resistance arises through an immune response preventing invasion or establishment of the pathogen within the host (Briese 1986). Shapiro and Robertson (1987) and Doane (1967) reported detection of PIBs in the adult tissues of gypsy moths examined by phase contrast microscopy. Bakhvalov et al. (1982) examined the hemocytes of sublethally dosed gypsy moth larvae, pupae, and adults and found that about 11% of the dosed individuals with infected larval hemocytes appeared to recover from infection before pupation having no evidence of infection in the pupal or adult stages. Those dosed individuals that survived to the adult stage contained no mature virus and

showed no reduction in fecundity. Similarly, Shields (1984) found no abnormalities nor any evidence of infection in adult gypsy moths that survived an LD_{50} dosage of NPV, administered in the larval stage, when tissues were examined by electron microscopy. Nor did she find any deleterious effects among survivors or their progeny in terms of survival, fecundity, pupal weight, development time, or susceptibility to NPV challenge, suggesting that survivors did not sustain productive infections in the adult stage and did not transmit disease to their progeny.

The relationship between the host and the virus can be affected by host developmental processes which may contribute to these apparently conflicting results. In general, resistance to viral pathogens increases with the age of the host which could result from the development of specific defense mechanisms against viral infection in older insects (Briese 1986). There is evidence that host developmental processes interfere with viral pathogenesis (Shapiro and Robertson 1987, Stairs 1965, Whitlock 1977). It is conceivable that hormonal or other host developmental changes render host cells semi- or nonpermissive for viral replication, making it difficult to detect virus by microscopic examination. Although the mechanisms involved are somewhat speculative, it appears that interactions between the host and pathogen during host metamorphosis affect the expression of the virus in the adult stage. Such

interactions may determine the ability of moths to transmit the virus to the subsequent generation.

Alternatively, viruses can be transmitted from one generation to the next via contamination of eggs with virus present in the environment rather than, or in addition to, maternal pathways (Wigley 1976). Viruses can persist in the soil and litter (Jaques 1970a, 1970b, 1975, Podgwaite et al. 1979, Weseloh and Andreadis 1986), or on tree bark (Doane 1975, Podgwaite et al. 1979, Weseloh and Andreadis 1986) for at least one year, and often longer. Viruses may be disseminated throughout the environment by rain or wind (Bird 1961, David and Gardiner 1967, Hofmaster 1961, Thompson and Steinhaus 1950). Although wind can act to transport viruses from soil reservoirs to plant surfaces, it may happen infrequently in forest ecosystems (Thompson 1978).

Rainfall appears to be a more important factor in the dissemination of viruses in forested habitats. Thompson (1978) demonstrated the effectiveness of rainfall in spreading Douglas-fir tussock moth NPV virus through the crowns of trees. In that study, the number of shoots contaminated with the virus rose from 18% to 100% following a rainfall. Rain was also shown to be responsible for dissemination of a sawfly NPV within the tree canopy (Bird, 1961). Rain is involved in leaching NPV from the environment onto eggs of the Douglas-fir tussock moth (Thompson 1978). It is not known whether precipitation

plays a role in environmental contamination of gypsy moth eggs. The mechanisms by which NPV becomes associated with egg masses, if understood, could possibly be exploited in the development of pest management strategies utilizing NPV as a biological control agent.

Factors Affecting Susceptibility of Insects to NPV

A factor which may affect the efficacy of insecticidal applications of pathogens is that susceptibility can vary widely among different populations of a single insect species (Martignoni and Schmidt 1961). Susceptibility to NPV among different gypsy moth populations can vary by more than 100-fold (Rollinson and Lewis 1973). These differences could be attributable to environmental factors such as temperature (Thompson 1959), light (Smirnoff 1967), or diet (Keating and Yendol 1987, Keating et al. 1988). Genetic differences may also play a role (Briese 1986, Ignoffo et al. 1985, Fuxa et al. 1988, Fuxa and Richter 1989, 1990, Reichelderfer and Benton 1974).

It has been suggested that cyclic fluctuations in insect population density result, in part, from genetic selection for resistance to disease (Myers 1990). Vasiljevic (1961) associated phases of gypsy moth outbreaks with changes in susceptibility to NPV, however, his conclusions might also be explained by fluctuating levels of

inoculum present in the environment. Although NPV prevalence generally increases with gypsy moth population density (Campbell 1963, Doane 1976a, 1976b), this might be due merely to increased transmission efficiency in dense host populations and an increasingly contaminated environment (Doane 1976a). Mathematical models, such as those of Anderson and May (1980, 1981), are based on the notion that populations consist of susceptible and resistant individuals. Identification of the factors which influence susceptibility and resistance of individuals and populations to NPVs, would improve the ability of such models to describe NPV epizootics, and to predict population collapses. Furthermore, in order to minimize or slow the development of resistance to NPV, it will be necessary to have an understanding of the relationships between gypsy moth population dynamics and susceptibility to the pathogen (Briese and Podgwaite 1985).

Vertical Distribution of Infected Larvae in Gypsy Moth Populations and Development of Sampling Protocol for Estimation of NPV Prevalence

To evaluate the efficacy of microbial insecticide treatments, it is necessary to estimate disease prevalence following application. Furthermore, estimates of the prevalence of naturally occurring NPV could be useful in

prediction of epizootics. However, sampling methods for estimating the prevalence of NPV in gypsy moth populations have not been standardized.

In order to develop an unbiased protocol for estimating the disease prevalence in a population the dispersion of infected individuals within a population must be known. In addition, the spatial arrangement of both infected and healthy hosts is important to disease transmission (Tanada 1963, Dwyer 1990). However, infection can affect larval behavior (Watanabe 1987) which can lead to altered dispersion of infected individuals. For example, the NPV disease of the nun moth, Lymantria monacha (L.), was originally termed 'Wipfelkrankheit' or 'tree-top disease' because infected larvae climb to the top of trees where they die (Benz 1963). Some entomopathogenic fungi (Carruthers and Soper 1987) can cause a similar behavior.

The effects of NPV infection on gypsy moth larval behavior are not well known. Doane (1967) reported that infected larvae become partially paralyzed prior to death, but the ultimate effect of this paralysis on larval movement was not examined. Lance (1985) found that infection did not affect the diel rhythm of gypsy moth larval feeding activity, though it is not clear how advanced infections were in these observations. Doane (1976a) suggested that the concentration of NPV-killed gypsy moth larvae in the upper crowns of trees may represent the normal distribution of both healthy and infected larvae, at least in the early

stadia, rather than disease-induced migration to the tree top.

Once the dispersion of infected individuals in a population is established, a sampling method is needed to estimate the prevalence of disease. However, sampling late stadium gypsy moth larvae can be difficult, because of their habit of resting in cryptic locations during the day (Forbush and Fernald 1896). Burlap bands stapled around tree trunks provide artificial resting sites readily used by late instars (Forbush and Fernald 1896). These bands have been used for controlling, detecting, and monitoring gypsy moth populations (McManus et al. 1980, McManus and Smith 1984). Burlap bands have also been utilized for estimating NPV prevalence (Webb et al. 1989, 1990) by counting the number of NPV-killed gypsy moth larvae found under the bands. Although, this technique is very simple, and therefore has great appeal, it is not known whether sampling from under burlap bands alone provides an unbiased estimate of disease prevalence. In fact, if infection affects the larval propensity to utilize burlap bands as artificial resting sites, the disease levels among larvae found under burlap bands are unlikely to be representative of disease prevalence in the population as a whole.

Objectives

The research described in this dissertation was undertaken in an attempt to gain an understanding of three critical aspects of gypsy moth NPV epizootiology: (1) the processes by which the pathogen is transmitted across host generations, (2) the role that population dynamics play in determining the susceptibility of first instars to disease, and (3) the behavior and vertical distribution of infected larvae in forested habitats and the relevance of these aspects to the development of sampling protocol.

CHAPTER 1

ENVIRONMENTAL CONTAMINATION OF EGG MASSES AS A MAJOR COMPONENT OF TRANSGENERATIONAL TRANSMISSION OF GYPSY MOTH NUCLEAR POLYHEDROSIS VIRUS

Introduction

Nuclear polyhedrosis virus epizootics have frequently been associated with the collapse of dense gypsy moth populations (Bess 1961, Doane 1970). Although NPV appears to be the key mortality factor regulating this economically important insect at high densities (Bess 1961, Campbell 1963, 1967, Doane 1970), the mechanisms by which gypsy moth NPV epizootics develop are not well understood. Earlier theories were built around the notion that latent virus, carried within the population, is activated by some environmental 'stress' factors, resulting in sudden, widespread virus-induced mortality (Aruga 1963, Bergold 1958, Steinhaus 1958, Vago 1963). This theory presumes that virus is transmitted within eggs produced by infected moths, a phenomenon which has been investigated (Wood et al. 1986), but has not been conclusively demonstrated for the gypsy moth or other Lepidoptera (Evans 1986).

Doane (1976b) proposed an alternative theory for the development of gypsy moth NPV epizootics, in which virus present at the beginning of a host cycle spreads in a

density-dependent manner throughout a population, rather than by spontaneous activation of latent virus. It was assumed that infected or contaminated females deposit virus externally (transovum) on eggs during oviposition. Recent studies support the idea that density-dependent processes are involved in the spread of inoculum throughout a gypsy moth population (Woods and Elkinton 1987). There is evidence from laboratory studies that infected females can transmit infective NPV to some progeny (Shapiro and Robertson 1987), but the extent to which such transmission occurs in the wild has not previously been examined.

Abiotic factors, such as rain and wind, have also been implicated in the dissemination of insect viruses throughout a population and its habitat (Bird 1961, Heimpel et al. 1973, Hofmaster 1961, Ossowski 1960, Thompson and Steinhaus 1950). Transgenerational transmission via persistence of inoculum in the environment have been shown for other insect viruses (Crawford and Kalmakoff 1977, Jaques 1970b, Mohamed et al. 1982). Gypsy moth larvae can acquire NPV by walking across contaminated substrates (Weseloh and Andreadis 1986), though the extent to which this mechanism contributes to transgenerational transmission of NPV in wild populations is not known. During a gypsy moth NPV epizootic, massive numbers of PIBs are released into the environment from the cadavers of virus-killed larvae which hang from tree stems and limbs. NPV has been shown to persist on bark for at least one year (Podgwaite et al. 1979). Whether rain is

important in leaching of NPV from the environment onto gypsy moth egg masses, as has been shown for Douglas-fir tussock moth (Thompson 1978) has not been established.

In this study I examined the roles of environmental contamination of egg masses and maternal transmission of virus in transgenerational transmission of gypsy moth NPV. The results of three experiments in which I (1) attempted to demonstrate vertical transmission by sublethally infected parents to progeny under field conditions, (2) examined the role of rainfall in NPV-contamination of gypsy moth egg masses subsequent to oviposition, and (3) compared the relative importance of environmental versus maternal effects in transgenerational transmission of NPV in two wild gypsy moth populations, are presented.

Materials and Methods

Three forested study sites were chosen on Cape Cod, Massachusetts. where the dominant tree species are black oak (Quercus velutina), scarlet oak (Q. coccinea), red oak (Q. rubra), white oak (Q. alba) and pitch pine (Pinus rigida) growing on sandy soils. In July 1986, gypsy moth population density was estimated at each site, by egg-mass counts in eight 6 m ('high-virus' and 'moderate-virus' sites) or twelve 10 m ('low-virus' site) diameter plots (Kolodny-Hirsch 1986).

These circular plots were uniformly distributed throughout each study site and their boundaries were marked using a 3 m or 5 m rope which pivoted around a center stake. As a crude indicator of the relative amount of NPV inoculum available for acquisition by egg masses, the number of gypsy moth larval cadavers hanging from tree stems and branches within the same 6 m or 10 m diameter plots were counted with the aid of binoculars. In 1986 the 'high-virus' site suffered heavy defoliation due to a dense gypsy moth infestation (8224 $\pm$ 2196 egg masses/ha) and an epizootic (24000 $\pm$ 9000 cadavers/ha) was observed. The 'moderate-virus' site suffered no noticeable defoliation and had a moderate gypsy moth infestation in 1986 (265 $\pm$ 94 egg masses/ha) which showed moderate levels of NPV mortality (177 $\pm$ 103 cadavers/ha). This site had harbored a moderate-density population for several years prior to 1986. The 'low-virus' site showed no noticeable defoliation, and supported a moderate gypsy moth infestation (255 $\pm$ 85 egg masses/ha), but there was no evidence of NPV mortality in the population in 1986 (0 cadavers/ha). The population density had been very low since 1984 when it was suppressed with an aerial application of *Bacillus thuringiensis*.

Maternal Dose Experiment

To look for evidence of maternal transmission of NPV from infected moths to eggs oviposited and overwintered in a natural habitat, larvae were fed a sublethal dose of NPV and survivors were mated and allowed to oviposit on trees in the 'low-virus' site. New Jersey Strain larvae (obtained from USDA Otis Methods Development Center, Otis ANGB, Massachusetts) were reared in an open room at 22°C (±3°), with ca. 10 larvae per cup, in 180-ml cups containing 85 ml of artificial diet (Bell et al. 1981). When the larvae were at late second stage, three quarters of the rearing cups were inoculated with NPV, by applying to the diet surface, 1 ml of a suspension of NPV (Gypchek), containing ca. 5×10^3 PIBs in sterile distilled water. The Gypchek was provided by the U. S. Forest Service, Hamden, Connecticut in a powder containing 5×10^{10} PIBs/g. Cups of undosed control larvae were reared in the same manner as the dosed larvae. Percentage virus-caused mortality in each cup was recorded at 24 days post-inoculation, when survivors and undosed controls began to pupate. Pupae were separated by sex and treatment and were placed into 24-oz cardboard containers for adult eclosion. Dosed females were mated with dosed males and undosed females were mated with undosed males. Half of the females from each group were deployed for oviposition in the 'low-virus' site by caging each female on the stem of a black oak tree with a smooth-sided 355-ml

waxed-paper cup placed over each female. The dosed and untreated moths were deployed onto different groups of trees. Egg masses were oviposited by dosed and undosed moths, onto 25 and 50 trees, respectively. The remaining half of each group were placed individually into clean 355-ml waxed-paper cups containing a strip of brown wrapping paper for oviposition. These undeployed egg masses were transferred into clean 30-ml plastic containers and were kept in the outdoor insectary until the following spring. In April 1987 each deployed egg mass was collected into a clean 30-ml plastic container by slicing the underlying bark from the tree. The blade used for this purpose was sterilized before moving to each new tree, with a 0.5% solution of sodium hypochlorite. All egg masses were bioassayed, by the following procedure which has been shown to be a good relative measure of the amount of inoculum associated with egg masses (Woods and Elkinton 1987). All eggs were hatched in the laboratory, and larvae from each egg mass were reared at 28°C ($\pm$ 2°C) and ca. 50% RH, in 180-ml cups containing 85 ml artificial diet. Forty to sixty larvae per egg mass were reared in groups of ca. 20 larvae per cup and were checked for NPV mortality after 7 and 14 days. At each observation, the number of virus-killed larvae was recorded and dead larvae were removed. Cause of mortality was judged on the basis of gross morphological appearance but questionable cadavers were examined under a light microscope for the presence of NPV

PIBs. After 14 days the percentage of larvae in each cup which had died due to NPV infection was recorded. Percentage of NPV-caused mortality among progeny of NPV-dosed and undosed parents was compared among larvae hatched from the deployed and undeployed egg masses.

Sheltering Experiment

To determine whether rainfall is instrumental in NPV-contamination of egg masses subsequent to oviposition, waterproof shelters were made of an inverted-V shaped piece of wax-impregnated cardboard, which was 21 cm long by 6 cm wide on each side. In August 1986, a shelter was stapled, at ca. 1.5 m above ground, to the northern aspect of the stem of each of 50 black oaks in both the 'high-virus' and the 'low-virus' sites. Each shelter was sealed tightly to the bark with weather-stripping and silicone caulking. Laboratory-reared female gypsy moths were mated with laboratory-reared males and then were deployed for oviposition on black oak trees in both sites, as described in the above experiment. On each of the 50 trees at each site, 3 egg masses were positioned directly above the shelter, where they were exposed to subsequent rainwater washing down the stem. In addition, 3 egg masses were positioned underneath each shelter, where they were protected from rainwater washing down the tree stems as verified by observations made during a rainstorm. Sheltered

egg masses were otherwise exposed to the same environmental conditions (such as temperature) as the exposed egg masses. Egg masses were deployed about one week after peak oviposition occurred in the wild populations. Control egg masses were not deployed onto trees, but were oviposited onto strips of paper in 355-ml waxed-paper cups and then were transferred into 30-ml plastic containers and were held in an outdoor insectary until the following spring. Within 3 days after oviposition, one sheltered and one exposed egg mass were collected from each tree at both sites by slicing off the bark on which the egg mass rested (August-collected). The blade was sterilized, in a 0.5% sodium hypochlorite solution, between each egg mass collection. Each egg mass and the underlying piece of bark was placed into a 30-ml plastic container. In late April 1987, the remaining sheltered and exposed egg masses were collected in the same manner (April-collected). All egg masses were held in the outdoor insectary until 27 April 1987, when they were brought into the laboratory, hatched, and bioassayed as described above.

Site-Switching Experiment

To examine the relative importance of environmental versus maternal effects in transmission of NPV across generations in wild populations the following experiment was

done. Female gypsy moth pupae were collected from the wild populations at the 'high-virus' site and at the 'moderate-virus' site. Pupae were placed into 24-oz cardboard containers, each containing ca. 20-30 pupae, which were held in the outdoor insectary until eclosion. Eclosed females from each population were deployed for oviposition in both sites in the following manner. Females from the 'high-virus' population were taken to the 'moderate-virus' site where each was caged with a resident wild male, in an inverted 355-ml waxed-paper cup stapled to the northern aspect of the stem of a black oak tree, for mating and oviposition. Males were captured in pheromone traps or as they aggregated around the cage used to transport females to field. Similarly, females from the 'moderate-virus' population were switched to the 'high-virus' site for mating and oviposition. For controls, females from each group were allowed to mate and oviposit in their site of origin (unswitched). An undeployed group consisted of 'high-virus' population females which were mated with laboratory-reared, New Jersey Strain males and were placed into clean 355-ml waxed-paper containers to oviposit on a strip of paper. Of the deployed egg masses, half of the switched and unswitched groups were collected within 3 days after oviposition (August-collected), by slicing off the bark on which each egg mass rested. The blade was sterilized with a 0.5% solution of sodium hypochlorite before moving to each new tree. Naturally occurring egg masses were also collected in

this manner from both populations. Each of the August-collected and undeployed control egg masses was placed into a 30-ml plastic container and held in the outdoor insectary until the following spring. The remaining site-switched and unswitched egg masses, as well as some additional naturally occurring egg masses from the 'high-virus' population, were collected in the April in a similar manner. I was unable to find any additional egg masses in the 'moderate-virus' site in the spring. In late April, all egg masses were brought into the laboratory to be hatched, and neonates were bioassayed as in the previous experiments. Percentage of virus-caused mortality was compared between the two deployment sites, the two maternal population sources, the undeployed controls, and the naturally occurring wild egg masses.

Data Analyses

All data were analyzed by ANOVA with SAS computer statistical package (SAS Institute, Cary, NC). Percentage of mortality values from each egg mass were transformed to arcsine square roots and analysis of variance was performed on the transformed proportions. Mean separations were determined by Sidak's option or by least square means with SAS GLM procedure (SAS Institute 1987).

Results

Maternal Dose Experiment

The mean (±SE) percentage NPV-induced larval mortality per cup of NPV-dosed parental stock was 17.9 (±4.9)%, but ranged from 0% to 100%. No NPV-induced mortality occurred among the undosed controls. Among the progeny of virus-dosed survivors and controls, oviposition site had the only significant effect on percentage of virus-caused mortality (Table 1.1, Figure 1.1). Mortality among all deployed egg masses was significantly greater ($P \leq 0.05$) than mortality among undeployed egg masses. NPV-caused mortality was not significantly different ($P = 0.94$) between progeny of virus-dosed versus undosed parents.

Sheltering Experiment

Deployment site was the most important factor contributing to NPV-induced mortality rates observed among neonates hatched from the egg masses in the sheltering experiment (Figure 1.2, Table 1.2). The percentage of NPV-induced mortality among neonates hatched from egg masses deployed in either site was significantly greater ($P = 0.0001$) compared with the undeployed controls or with each other ($P = 0.0001$).

Mortality did not differ with exposure (P = 0.30) or collection date (P = 0.10). In the 'high-virus' site, both sheltered and exposed egg masses showed 11-16% less mortality among larvae from August-collected compared with April-collected egg masses, but that difference was not significant (P = 0.11).

Site-Switching Experiment

In this experiment, deployment site had the most important effect on NPV-caused mortality among hatching neonates (Table 1.3, Figure 1.3). Mortality from all egg masses deployed in the 'high-virus' site was significantly greater (P = 0.006) than mortality from those deployed in the 'moderate-virus' site or from the undeployed progeny of 'high-virus' females. Mortality among larvae from egg masses deployed in the 'moderate-virus' site was not significantly different (P > 0.05) than among larvae from the undeployed control egg masses.

NPV mortality from egg masses oviposited by unswitched deployed females was not significantly different (P > 0.40), when compared by collection date, than mortality among larvae hatched from naturally occurring egg masses in either site. Therefore, I am confident that the deployment procedure itself did not skew the results.

Collection date was not a significant factor (P = 0.18) although there was a trend of less NPV-caused mortality among larvae from August-collected egg masses compared with those collected in April. Therefore, egg masses may have acquired some additional inoculum over the winter but the effect was small compared with the deployment site effect.

When data were analyzed separately by deployment site, a maternal population effect was evident among egg masses deployed in the 'high-virus' site, but not among those deployed in the 'moderate-virus' site. In the 'high-virus' site, progeny of 'high-virus' population females showed significantly (P = 0.05) greater virus-caused mortality compared with progeny of 'moderate-virus' population females.

Discussion

Transmission of virus from one generation to the next could occur by larval ingestion of inoculum persisting in the environment from the previous generation, or by acquisition of NPV by eggs or egg masses. Neonates may acquire virus by walking across contaminated substrates (Weseloh and Andreadis 1986, Woods et al. 1989), but the importance of such a mechanism in transgenerational transmission of NPV in gypsy moth populations is not known.

Primary inoculum carried by gypsy moth egg masses is an important component in the development of an epizootic (Doane 1969, 1976a, Woods and Elkinton 1987). Neonates can become infected upon hatch due to ingestion of virus present on the egg mass surface (Doane 1969, 1970). The mechanisms by which NPV is transmitted to egg masses have not been fully identified. Three possible ways in which egg masses might acquire NPV are (1) transmission by infected or externally contaminated moths to progeny, (2) environmental contamination, or (3) passive transport by parasitoids.

Parasitoids can transport NPV to larval hosts (Levin et al. 1979, Raimo et al. 1977), but the ability of egg parasitoids such as Oencyrtus kuvanae and Anastatus disparis to mechanically transmit NPV to gypsy moth eggs has not been examined.

Although much of the early literature suggested that viruses are readily transmitted within the egg (Longworth and Cunningham 1968, Swaine 1966), various laboratory tests have failed to conclusively demonstrate transovarial transmission for any of the Lepidoptera (Evans 1986, Wood et al. 1986). Doane (1969) suggested that infected gypsy moths may transmit virus transovum to the egg surface, rather than within the egg. Shapiro and Robertson (1987) showed a positive correlation between the occurrence of NPV in gypsy moth females and NPV-induced mortality rates among their progeny, providing support for maternal transmission, but

they did not attempt to distinguish between transovarial or transovum transmission.

I failed to demonstrate either mode of vertical transmission from sublethally dosed parents to progeny. Undosed females and females fed a dose of NPV both produced offspring that suffered less than 2% NPV-caused mortality, when the eggs were oviposited onto a clean surface. Although every precaution was taken to avoid accidental contamination of the adults and egg masses, I suspect that the small amount of NPV-caused mortality observed in those samples was due to inadvertent contamination, and not transovum or transovarial transmission, since the same low infection rate occurred among progeny of both dosed and undosed parents. It should be noted, however, that I examined progeny of dosed parents which suffered only a mean of 17% NPV-caused mortality. These low parental mortality rates may explain why I did not find any evidence for transmission of virus by NPV-dosed adults to progeny. However, Shields (1984), also compared gypsy moth survivors of an LD_{50} dose of NPV, and their progeny, with control adults and progeny in terms of several developmental, histological, and physiological parameters, and did not find any evidence of infection in surviving adults or transmission of virus to progeny. Shapiro and Robertson (1987) found very low mortality rates (4.7%) among progeny of parents fed an LC_{20} dose and only slightly higher mortality rates (11.5%) among progeny of parents fed an LC_{80}

dose. In contrast, I observed mortality rates ranging from 20% to 46% among egg masses deployed in the 'high-virus' site. It is possible that oviposition in a natural habitat, as opposed to an artificial substrate, also accounted for some of the differences between my results and those of Shapiro and Robertson (1987), although I also found no evidence of maternal transmission when eggs were oviposited into plastic cups.

The results of the site-switching experiment, however, may provide some evidence for maternal transmission, as 10% NPV-mortality occurred among undeployed progeny (oviposited into clean cups) of 'high-virus' females. This was not significantly different from the mortality rates among progeny of either parental population deployed in the 'moderate-virus' site. Either transmission by infected moths or mechanical transfer of NPV by externally contaminated moths could explain this result. Other studies have shown that eggs can acquire virus externally via passage through contaminated genitalia (Elmore and Howland 1964, Martignoni and Milstead 1962, Tatchell 1981). During an epizootic massive numbers of PIBs are released from disintegrating cadavers of virus-killed larvae. Genitalia and/or abdominal setae of female moths could become environmentally contaminated with virus, which could be subsequently transferred externally to eggs. 'High-virus' adults used in the site-switching experiment were collected as pupae from silken debris mats and other heavily

contaminated habitats, and were allowed to eclose in groups of 20-30, therefore it is quite possible that moths became externally contaminated at eclosion. It is difficult to conclude whether the relatively low rate of polyhedrosis seen among the progeny of undeployed moths resulted from transmission by infected moths or whether it merely represents transfer of NPV from externally contaminated females to their progeny.

In addition, progeny of 'high-virus' females showed 25% greater NPV-caused mortality, compared with progeny of 'moderate-virus' females, when both were deployed in the 'high-virus' site. Although some inoculum may have been transmitted by externally contaminated or infected 'high-virus' moths, that does not entirely explain the large difference seen between progeny of the two populations, as there was no difference between the two groups when both were deployed in the 'moderate-virus' plot. This could be due to differential susceptibilities of the two populations to NPV, or perhaps to a synergistic, rather than additive effect of NPV from parents as well as the environment. This apparent maternal effect was statistically significant but it was based on a limited number of egg masses (42 from 'high-virus' females, 8 from 'moderate-virus' females). Consequently, the effect should be verified with further experiments before too much weight is placed on this result.

In contaminated sites, it appears that the major route through which egg masses acquire NPV is via environmental

contamination, rather than by direct transmission by contaminated or infected mothers. I found high NPV-caused mortality rates among progeny of undosed, laboratory-reared moths deployed in the 'high-virus' site (mean = 42.5%) (sheltering experiment) as well as among deployed or naturally occurring progeny of 'high-virus' moths deployed in the 'high-virus' site (site-switching experiment).

The amount of inoculum acquired may be related to the amount present in the environment. The incidence of NPV infection in a population is density-dependent (Doane 1976a). Presuming that NPV PIBs released from the cadavers of virus-killed larvae contribute significantly to the pool of inoculum available for acquisition by egg masses, then the amount of inoculum available would be expected to be related to population density and NPV mortality levels in the parental generation. Such a trend was evident in my experiments. Egg masses deployed in the 'high-virus' site, where an NPV epizootic had occurred in the dense natural population, showed 20-46% NPV-caused mortality in the bioassay. By comparison, there was only 4-5% mortality among egg masses deployed in the 'low-virus' site. NPV-induced mortality levels were intermediate (13-14%) among larvae hatched from egg masses deployed in the 'moderate-virus' site. This was not significantly different from mortality of the undeployed progeny of females from the 'high-virus' population, which suggests that the amount of

inoculum acquired from the environment in the 'moderate-virus' site was small.

Because local wild males were mated with the site-switched females in the site-switching experiment, it might be argued that the large differences in mortality between the deployment sites were actually due to a paternal effect rather than an environmental one. However, a similar deployment site effect was also seen in the Sheltering and Maternal Dose experiments which could not be attributed to a paternal effect (all the males were laboratory-reared and were from the same strain regardless of deployment site). Therefore, it is much more likely that the differences between sites in the site-switching experiment also were due to environmental as opposed to paternal effects.

Rainfall does not appear to play a role in leaching NPV from the environment into egg masses. However, rain may be involved in spreading NPV throughout the population and its environment prior to oviposition, as has been shown for other systems (Bird 1961, Ossowski 1960). The shelters were erected only a few weeks prior to deployment of egg masses; therefore, I cannot rule out that possibility.

In all three experiments, egg masses acquired very little additional NPV after oviposition, even when they remained in the environment over the entire 8-month period between oviposition and hatch. Because NPVs are rapidly inactivated by ultraviolet radiation (Young and Yearian 1974), it is possible that any virus introduced onto egg

masses over the winter was rendered inactive by exposure to sunlight, although I attempted to reduce such a possibility by deploying the egg masses only on the northern aspect of each tree. Because most NPV was acquired rapidly (within 3 days of oviposition) from the habitat, I suspect that the inoculum was acquired from the substrate during oviposition. Perhaps PIBs are incorporated from the substrate into the egg mass, as the moth rubs her abdomen laterally on the substrate to deposit her abdominal setae on the eggs during oviposition.

Wind has been implicated as a factor responsible for movement of virus throughout certain environments (Young and Yearian 1986). Soil is well known as an important reservoir of many insect viruses, where they are afforded a certain amount of protection from inactivation by ultraviolet light (Jaques 1970a, 1970b, 1975, Podgwaite et al. 1979, Weseloh and Andreadis 1986). Wind may be important in movement of virus from soil to vegetation (David and Gardiner 1967). Perhaps some virus was carried on dust particles in the air, and was blown onto the surface of egg masses during winter and spring storms, which might account for the slight increase in mortality seen from April-collected (those that overwintered on trees), compared with fall-collected egg masses. Such a phenomenon did not appear to be important in these experiments as the amount of inoculum acquired over the winter was not significant.

The results of this study, as well as those of Shapiro and Robertson (1987), indicate that vertical transmission of infective NPV from infected or externally contaminated gypsy moths to their progeny occurs only to a limited extent, or perhaps only above some threshold parental infection level. Alternatively, it is possible (though there is no direct evidence) that transovarially transmitted virus remains latent in many progeny. Maternal transmission is perhaps more important in years following an epizootic, after weathering has reduced environmental inoculum to very low levels, as a means for re-introducing low levels of virus into an increasing population, thereby initiating the density-dependent spread of inoculum through the population and the environment. Environmental contamination of egg masses was readily apparent in all three experiments, and was substantial among egg masses oviposited into the site in which an epizootic had occurred in the same generation. This factor appears to be an important component of transmission of gypsy moth NPV across generations.

Table 1.1. ANOVA for main effects and interactions of parental dose treatment and deployment site on percentage of NPV-caused mortality among progeny of NPV-dosed or undosed gypsy moths which were deployed onto trees or were oviposited into clean plastic cups (undeployed). Proportions were transformed to arcsine square roots.

SOURCE	df	SS	F	Prob > F
Parental Dose Treatment	1	0.000244	0.01	0.9406
Deployment Site	1	0.168863	3.85	0.0513
Parental Treatment x Deployment Site	1	0.002428	0.06	0.8143
Error	182	7.985742		

Table 1.2. ANOVA for main and crossed effects of deployment plot, exposure (sheltered versus exposed), and collection date (April versus August) on percentage of NPV-caused mortality among gypsy moth neonates hatched from egg masses deployed in the 'high-virus' and 'low-virus' sites. Proportions were transformed to arcsine square roots.

SOURCE	df	SS	F	Prob > F
Deployment Site	2	143.2639	71.63	0.0001
Exposure	1	1.0567	1.06	0.3050
Site x Exposure	1	0.9332	0.93	0.3350
Collection Date	1	2.7577	2.76	0.0981
Site x Collection Date	1	2.2384	2.24	0.1359
Exposure x Collection Date	1	0.0078	0.01	0.9295
Site x Exposure x Collection Date	1	0.0937	0.09	0.7597
Error	242	1.0000		

Table 1.3. ANOVA for main effects and interactions of deployment plot, maternal population, deployment treatment (deployed versus naturally occurring), and egg mass collection date on percentage of NPV-caused mortality among deployed, undeployed, or naturally occurring progeny of gypsy moths switched or unswitched between an 'high-virus' site and a 'moderate-virus' site. Egg masses were collected in August, within 3 days of oviposition, or in April of the following spring. Proportions were transformed to arcsine square-roots.

SOURCE	df	SS	F	Prob > F
Deployment Site	2	1.596349	5.29	0.0061
Maternal Population	1	0.474493	3.14	0.0783
Deployment Treatment	1	0.104481	0.69	0.4067
Collection Date	1	0.262497	1.74	0.1893
Site x Maternal Population	1	0.320290	2.12	0.1473
Site x Collection Date	1	0.017056	0.11	0.7372
Maternal Population x Collection Date	1	0.060418	0.40	0.5279
Site x Maternal Population x Collection Date	1	0.000000	0.00	0.9993
Error	98	13.926266		

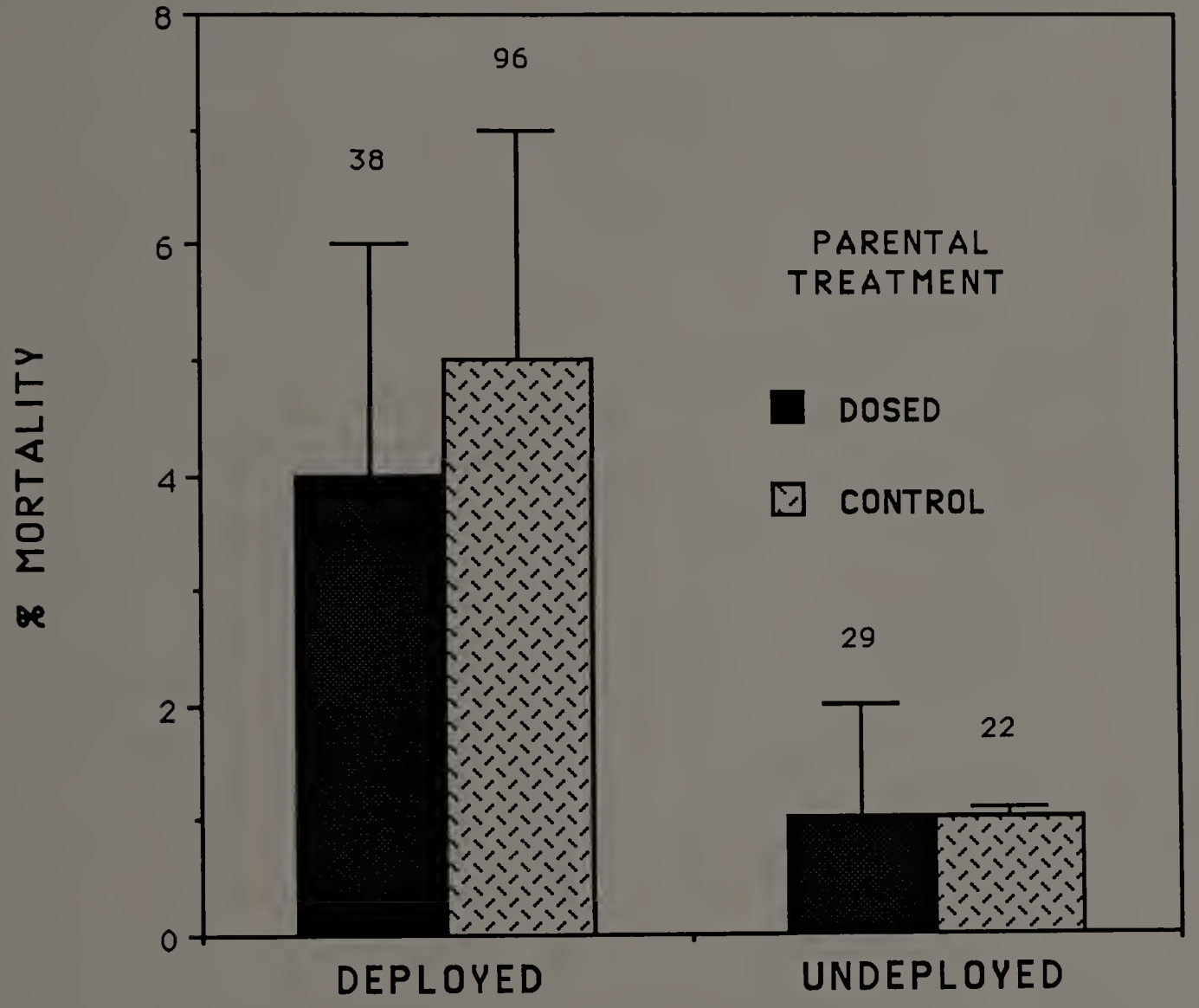


Figure 1.1. Mean percentage of NPV-caused mortality among progeny of NPV-dosed and control (undosed) gypsy moths deployed onto trees in the 'low-virus' site and undeployed dosed and undosed moths which oviposited into clean cups. Error bar = $\pm$ 1 SE of the mean. Numbers atop each column represent the number of egg masses included in the mean. Columns topped by the same letter are not significantly different (Sidak's inequality test, $P \leq 0.05$).

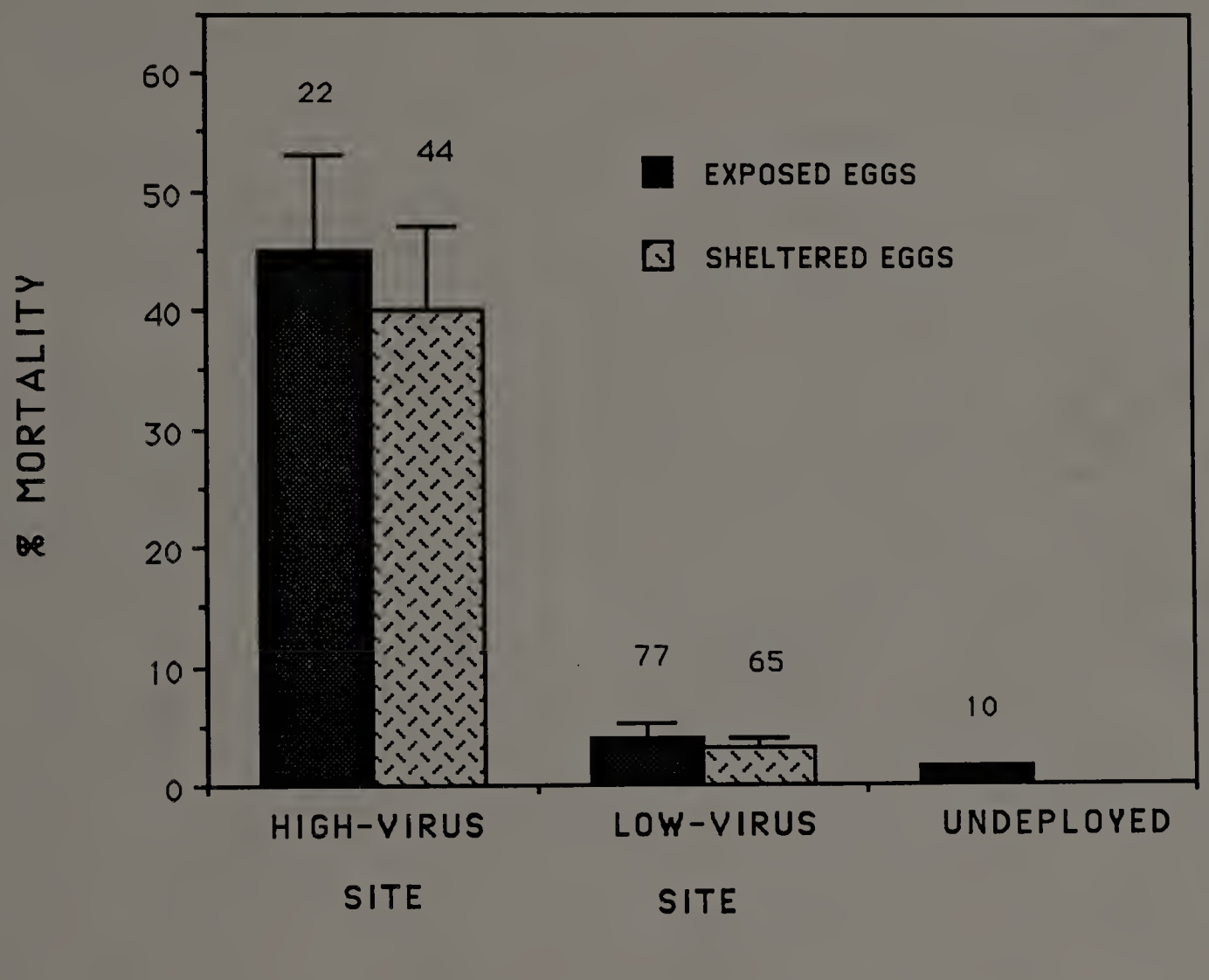


Figure 1.2. Mean percentage of NPV-caused mortality among neonates hatched from sheltered and exposed egg masses which were deployed in 'high-virus' and 'low-virus' sites. Undeployed egg masses were oviposited into clean plastic cups. August-collected and April-collected egg masses combined within each exposure and deployment plot. Error bars = $\pm$ 1 SE of the mean. Numbers atop each column represent the number of egg masses included in the mean.

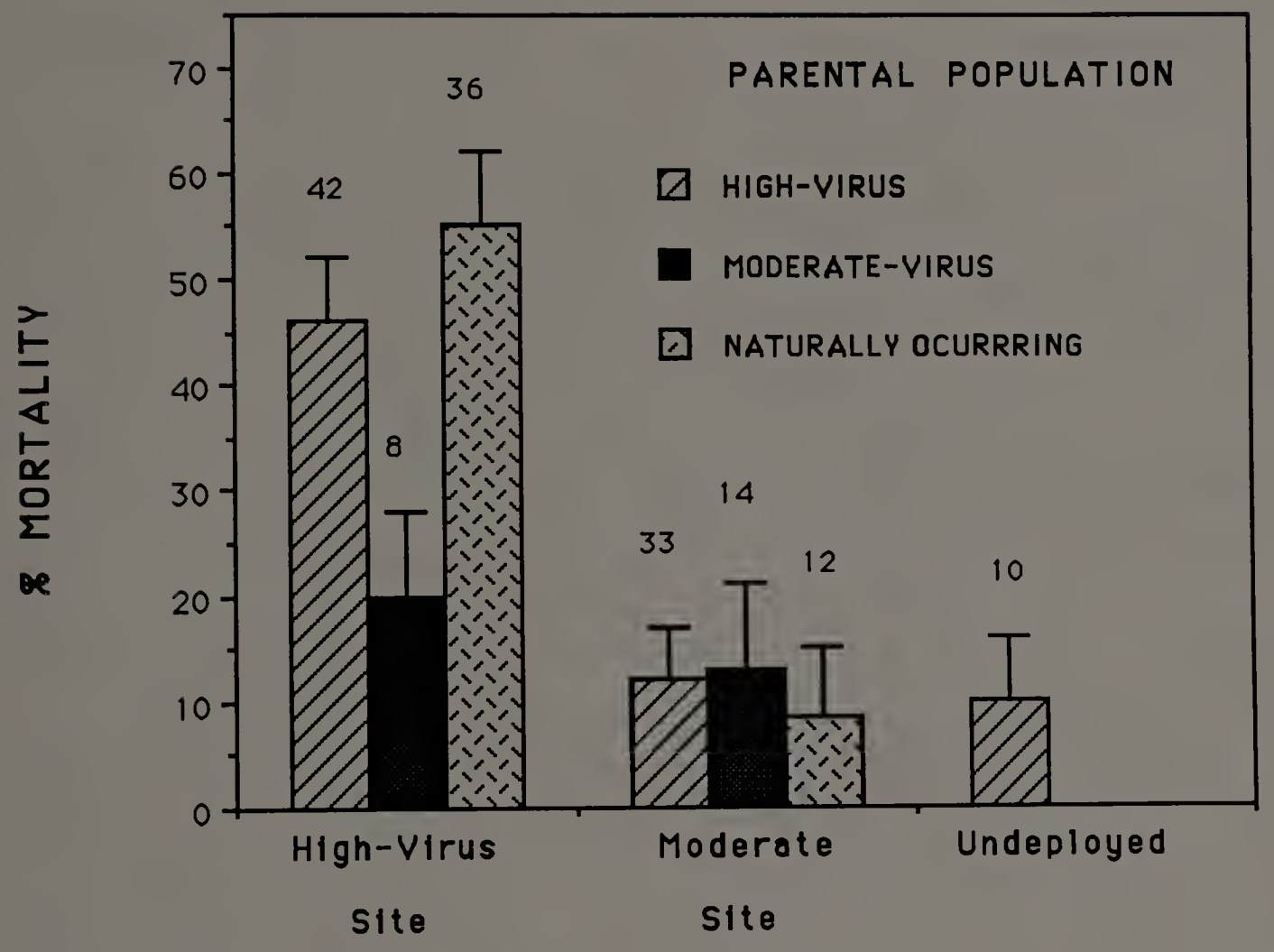


Figure 1.3. Mean percentage of NPV-caused mortality among neonates hatched from egg masses oviposited by gypsy moths collected from 'high-virus' and 'moderate-virus' populations which were site-switched or unswitched and deployed for oviposition in 'high-virus' and 'moderate-virus' sites, among neonates hatched from naturally occurring egg masses collected from each site, and among those oviposited by undeployed 'high-virus' moths into clean cups. Error bars = $\pm$ 1 SE of the mean. Numbers atop each column represent the number of egg masses included in the mean.

CHAPTER 2

TRANSMISSION OF NUCLEAR POLYHEDROSIS VIRUS TO GYPSY MOTH EGGS VIA CONTAMINATED SUBSTRATES

Introduction

Infection of first instars is instrumental in the development of NPV epizootics (Doane 1969, 1970, 1976a, 1976b, Woods and Elkinton 1987). Doane (1969, 1970, 1975) showed that a major source of infection for newly hatched larvae is the overwintering stage, the eggs, which are deposited in masses in late summer. Leonard and Doane (1966) and Doane (1969, 1975) demonstrated that chemical disinfection of the surface of gypsy moth eggs almost entirely eliminated NPV-caused mortality among larvae hatched from the eggs, indicating that virus is carried on the surface of the egg. Upon hatching, first instars ingest NPV along with pieces of the egg chorion and egg mass setae (Doane 1969, 1975, 1976a). But the mechanisms by which eggs become contaminated with NPV are not well understood.

Theoretically, eggs can become infected or contaminated with virus by two means: by vertical transmission, virus transmitted by infected or externally contaminated moths directly to their progeny; or by horizontal transmission, by environmental contamination of the egg mass.

Doane (1976a) and Podgwaite et al. (1981) suggested that NPV is transmitted transovum (ie. on the surface of the egg) by infected female moths to the eggs. Shapiro and Robertson (1987) reported in a laboratory study that infected female moths may transmit virus to offspring. They found 11% NPV-induced mortality among progeny of gypsy moths surviving an LD_{80} dose of NPV. It was not determined whether transmission was transovum or transovarial (within the egg). However, as described in Chapter 1, I was unable to demonstrate transmission of NPV from sublethally dosed females to their progeny under field conditions, although I used lower doses of NPV to infect the parental stock than did Shapiro and Robertson.

Evans (1986) has argued that conclusive evidence for transovarial transmission of NPV in Lepidoptera is lacking and suggests that in this group maternal transmission of NPV to eggs is the result of environmental contamination of the female rather than internal infection. I found that females surviving an NPV epizootic produced progeny which suffered a relatively low rate of NPV-induced mortality (10%) when oviposited into a clean environment (Chapter 1). However, mortality rates were high (20-55%) among progeny of both wild and laboratory-strain moths when eggs were oviposited onto trees located in sites in which an epizootic had occurred, suggesting that eggs acquired most NPV directly from environmental rather than maternal sources. Furthermore, contamination of the eggs occurred during or

shortly after oviposition. Eggs sampled within three days of oviposition had the same amount of inoculum as those that overwintered on forest trees (Chapter 1).

I proposed that in NPV-contaminated environments (eg. after an epizootic) gypsy moth eggs become contaminated with NPV from the substrate on which they are deposited. In the study reported here I examined the transfer of NPV from artificially contaminated substrates to eggs and the subsequent infection and NPV mortality among first instars.

Materials and Methods

In August 1987, after adult emergence, I felled three black oak (Q. velutina) trees from a site on Cape Cod, Mass., where a resident gypsy moth population suffered a moderate level of NPV infection. Peak prevalence of NPV during the larval period was 20%. The bole of each tree was cut into four 1-m long bolts, and each of the twelve bolts was nailed upright onto a square fiberboard base. Each of three treatments was randomly assigned to a group of four bolts. Three treatments were applied: (1) the entire bark surface was sprayed until drenched with 10% chlorine bleach to inactivate any NPV present, (2) bark surface was drenched with an NPV suspension containing 5×10^8 PIB per ml water (Gypchek provided by the U.S. Forest Service, Hamden, Connecticut), or (3) bolt remained untreated (control).

Newly eclosed New Jersey Strain gypsy moths were obtained from a laboratory colony (provided by the U. S. Department of Agriculture Otis Methods Development Center, Otis ANGB, Massachusetts). Male and female moths were caged in pairs in inverted 355-ml waxed-paper cups stapled onto the bark surface of each bolt. Females mated and subsequently oviposited 12-18 egg masses onto each bolt for a total of 63, 50, and 58 egg masses in the bleach-treated, NPV-treated, and untreated control treatments, respectively. The bolts with the egg masses on them remained in an outdoor screened insectary until the following spring.

In April 1988, each egg mass was gently scraped from the bolt into a clean 30-ml covered plastic cup using a knife blade disinfected with 10% chlorine bleach. Approximately half of the egg masses within each treatment were divided into an inner half (containing those eggs proximal to the underlying bark surface), and an outer half (eggs distal to the bark surface). All eggs were refrigerated at 10°C for 20 days after which they were placed in an incubator at 29°C to hatch.

I did bioassays of egg masses by the following procedure which has been shown to be a good measure of the relative amount of inoculum associated with egg masses (Doane 1976a, Woods and Elkinton 1987). Approximately 20 newly hatched first instars were randomly selected from each egg mass (whole egg masses) or each half egg mass (divided egg masses) and were transferred to 180-ml cups containing

85 ml of artificial diet (Bell et al. 1981). Larvae were reared for 14 days at 27°C (± 1°C) and ca. 50% RH. The photoperiod was 16:8 (L:D). Larvae were checked after 7 and 14 days for NPV mortality. Secondary infection within cups prior to 14 days is minimal under these rearing conditions (Woods and Elkinton 1987). At each observation the number of virus-killed larvae was recorded and dead larvae were removed. Cause of mortality was judged on the basis of gross morphological appearance but questionable cadavers were examined under a compound microscope for the presence of PIBs. After 14 days the total number of larvae in each cup which had died due to NPV infection was recorded. The mean percentage of NPV-caused mortality was compared among larvae hatched from egg masses from the three bolt treatments and from eggs from the inner versus outer portions of the egg masses.

Percentages were converted to arcsine square roots and were analyzed by ANOVA using the computer statistical package SAS PROC GLM (SAS Institute 1987). Treatment mean separations at the P=0.05 level were determined by Sidak's test for inequality (SAS Institute 1987).

Results and Discussion

The results are shown graphically in Figure 1. Larvae that hatched from egg masses oviposited onto the NPV-treated

bolts showed significantly higher ($P \leq 0.05$) NPV-caused mortality compared with those hatching from egg masses laid on either the bleach-treated or the untreated bolts. The mortality rate among larvae hatched from eggs from the bleach-treated bolts was not significantly different ($P \leq 0.05$) than those hatched from eggs laid on the untreated bolts.

Among the untreated and bleach-treated groups the mean mortality rate did not vary significantly ($P = 0.73$, untreated; $P = 0.36$, bleach-treated) with position of eggs in the mass (inner versus outer). However, significantly greater ($P = 0.01$) NPV-induced mortality was recorded among larvae hatched from inner eggs compared with larvae hatched from outer eggs when egg masses were oviposited onto NPV-treated bolts.

Environmental persistence of NPV may be critical for maintenance of the pathogen in low density populations in the years following an epizootic (Doane 1976a, Podgwaite et al. 1979, Weseloh and Andreadis 1986). First instars that traverse a virus-contaminated surface can become infected (Weseloh and Andreadis 1986, Woods et al. 1989), presumably by transfer of virus to food where it is ingested. However, the importance of this mechanism to transmission of NPV across generations in natural populations is not known. Alternatively, there is much evidence that environmental contamination of egg masses is an important mode of transmission of the virus from one generation to the next

for gypsy moth (Doane 1970, 1975, 1976a, Woods and Elkinton 1987, Chapter 1) as well as for other Lepidoptera (Wigley 1976, Thompson 1978). The results of this study indicate that egg masses can become contaminated upon oviposition on contaminated substrates. This is supported by a similar finding that egg masses laid on tree trunks have significantly higher amounts of NPV inoculum than do egg masses deposited onto other substrates such as rocks, understory vegetation, and branches in the same site (Woods et al. 1990).

Acquisition of NPV from contaminated substrates by egg masses could help explain why gypsy moth populations often remain at low density after an epizootic (Doane 1976a). During an epizootic, NPV-infected larvae die in large numbers on tree trunks, thereby contaminating the bark with PIBs. Bark flaps and crevices on tree boles are among favored oviposition sites (Bess 1961, Campbell et al. 1975). Because NPV can persist on bark for at least one year (Doane 1975, Podgwaite et al. 1979, Weseloh and Andreadis 1986), many oviposition sites are likely to be heavily contaminated with NPV following an epizootic. It is apparent from this study that egg masses oviposited on NPV-contaminated bark substrates readily become contaminated with the virus.

I found that the innermost eggs (those in direct contact with the substrate) produced larvae about twice as likely to die from NPV compared with larvae hatched from eggs in the outermost part of the egg mass. Doane (1975)

found that the setae deposited into the egg mass from the abdomen of the female during oviposition are a good source of NPV inoculum for newly hatched larvae. It is likely that virus from the substrate is incorporated into the egg mass, especially onto those eggs deposited into the first (inner) layers, as the female repeatedly moves her abdomen back and forth on the substrate while depositing eggs and her abdominal setae into the egg mass. The outer layers would not become contaminated with as much inoculum because neither the eggs nor the female's abdomen are in direct contact with the substrate during oviposition.

It is also possible that NPV was initially dispersed throughout the egg mass but was either inactivated by sunlight, pH, or microbial degradation, or physically removed by other environmental factors (such as rain) in the outer exposed portion of the egg mass. However, because the bolts were somewhat protected from sunlight and wind and were almost completely protected from precipitation in the screened insectary, I believe it is unlikely that much environmental degradation of NPV in the outer portions of the egg mass occurred. This is supported by the data presented in Chapter 1 showing that the amount of inoculum associated with egg masses three days after oviposition was not significantly altered after overwintering on forest trees.

Perhaps the most effective location for virus to overwinter is on the surface of eggs in the inner portions

of the egg mass. As pointed out by Evans (1986), persistence of viruses outside the host, which is the most common means of transmission from one period to another, is dependent on the quantity of virus remaining viable and the probability of the inoculum being encountered by susceptible hosts. Inner eggs and the virus associated with them are somewhat protected from predation, parasitism and environmental damage by the thick layer of outer eggs and setae overlaying them. Therefore, it is highly probable that inner eggs and the virus present on them will both remain viable over the winter, thereby increasing the likelihood that larvae hatching from these eggs will become infected the next season.

It has been suggested that the primary source of inoculum for eggs and first instars is maternal (Doane 1976a, Podgwaite et al. 1981). Although I cannot rule out the possibility that maternal transmission occurs, the results of this study, in conjunction with the data presented in Chapter 1, indicate that environmental contamination of the oviposition substrate may also be an important route of transmission to eggs, at least in contaminated environments.

This mechanism of transgenerational transmission may also have important implications for management of the gypsy moth. Dispersal of infected first instars from contaminated egg masses can serve to introduce NPV from a contaminated area into uninfected populations or to augment the disease

level in a moderately diseased population (Bogenschutz et al. 1989). Current suppression strategies that use insecticidal formulations of NPV are aimed at spraying early instars and the results of these applications have often been disappointing (Lewis and Yendol 1981, Podgwaite 1985). However, because the virus appears to be protected from environmental degradation within the egg mass, perhaps trees can be sprayed with NPV prior to oviposition in the late summer. Such a practice might serve to augment the prevalence of naturally occurring disease among early instars the following spring, thereby taking advantage of secondary spread of the virus through the population to initiate an epizootic.

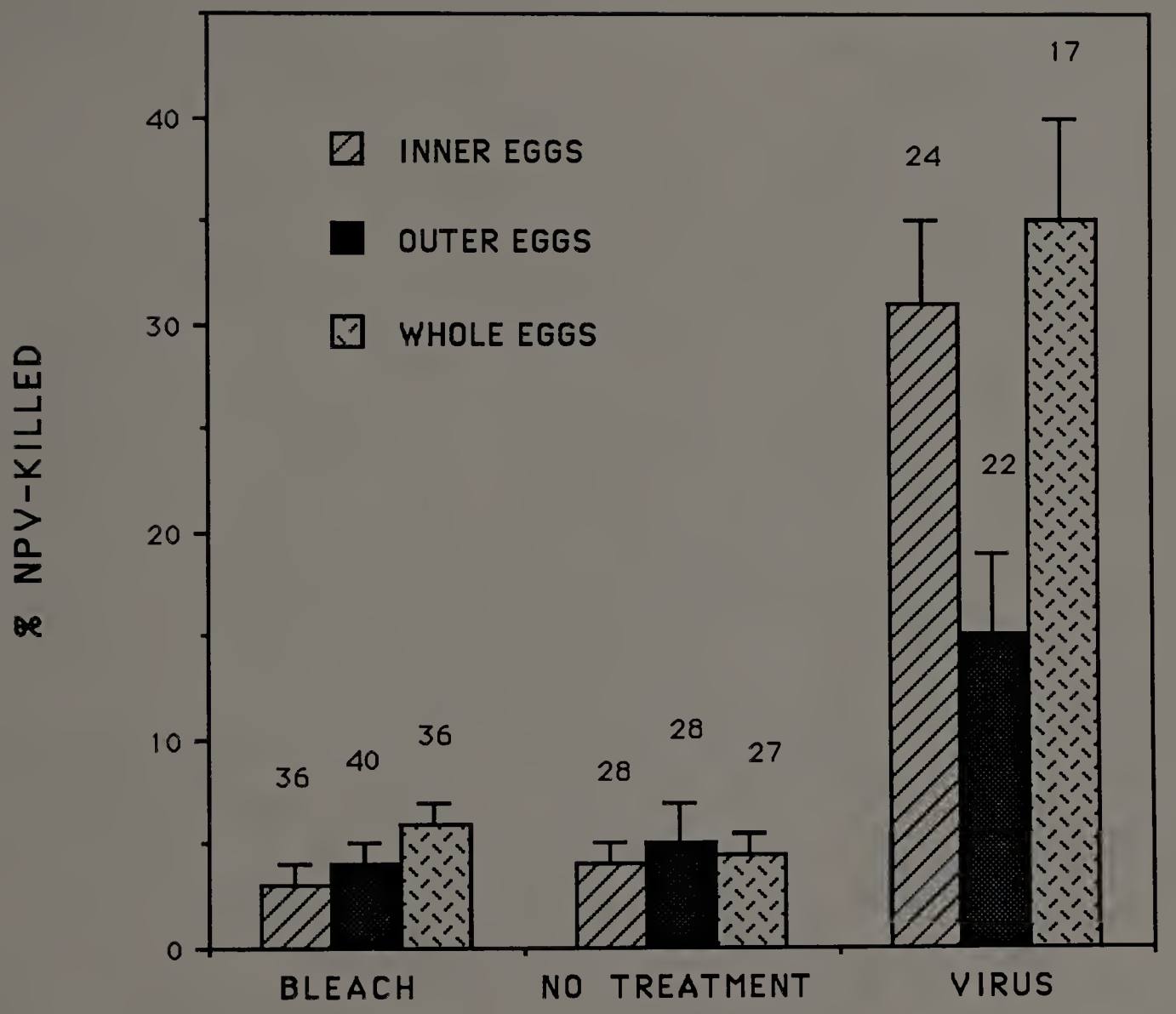


Figure 2.1. The percentage of gypsy moth larvae that died because of NPV infection after hatching from whole egg masses and the inner (proximal to bark substrate) and outer (distal to substrate) portions of divided egg masses that were laid on NPV-treated, bleach-treated, or untreated bolts cut from black oak stems. Error bars represent + 1 standard error of the untransformed mean. Numbers atop each bar represent the number of egg masses in each treatment.

CHAPTER 3

THE EFFECT OF GYPSY MOTH METAMORPHOSIS ON THE DEVELOPMENT OF NUCLEAR POLYHEDROSIS VIRUS INFECTION

Introduction

As shown in Chapters 1 and 2, it is apparent that, at least in contaminated habitats, environmental contamination of egg masses resulting from oviposition on contaminated substrates is the primary means by which NPV is transmitted to eggs. However, on the basis of these experiments the possibility that maternal transmission occurs cannot be ruled out. Such a mode of transgenerational transmission might be critical to maintenance of NPV in populations during periods of low host density and disease prevalence.

Shapiro and Robertson (1987) showed that NPV-caused larval mortality in the parental generation of gypsy moth was associated with NPV-induced mortality among progeny, suggesting that virus persisted in adult tissues and was vertically transmitted to eggs. However, I was unable to demonstrate transmission of NPV to eggs produced by survivors of NPV challenge (Chapter 1). Evans (1986) has suggested that transmission of NPV by adult Lepidoptera to progeny in the field may be limited to mechanical transfer of virus from externally contaminated adults to eggs. It appears that complex interactions between pathogen and host

may operate during host metamorphosis which affect the expression of the virus in pupal and adult host tissues. Clearly, these interactions are important to the ability of the host to transmit infective NPV to the subsequent generation, a phenomenon which could be of great importance to the utilization of these pathogens in pest management programs.

In an effort to elucidate the development of NPV infection prior to, during, and subsequent to host metamorphosis, gypsy moth larvae, pupae, and adults were examined with a labelled DNA probe to determine if low levels of NPV can be detected in survivors of sublethal infection.

Materials and Methods

Larvae and Virus

New Jersey Strain gypsy moth eggs were obtained from the USDA Otis Methods Development Center, Otis ANGB, Massachusetts and were reared on artificial diet (Bell et al. 1981) at 27°C (± 1°C), 50-70% RH and a 16:8 hr (L:D) photoperiod.

NPV clone 'g' (Smith et al. 1988), was propagated *in vivo* and purified according to the methods of Stiles et al. (1983).

DNA Hybridization Detection of NPV in Sublethally Dosed Survivors

Newly molted fourth and fifth instars (the 'early-dosed' group) were starved for 48 hr following ecdysis and then each imbibed a 10 ul droplet of sterile distilled water (control) or a virus suspension containing ca. 7, 15, 75, 1.5×10^2, 1.5×10^3, 1.5×10^4, or 1.5×10^5 PIB, resulting in no virus-caused mortality among the 37 control fourth instars and 90 control fifth instars and 0-91% virus-caused larval mortality among 170 virus-treated fourth instars and 468 virus-treated fifth instars. A second group of larvae, dosed in the ultimate stadium 4-9 days prior to pupation ('late-dosed'), would not readily ingest a droplet even after 48 hr starvation, therefore, virus was administered by contaminating a diet disk (3 x 6 mm) with a 10 ul droplet containing 1.7×10^4 or 1.7×10^5 PIB, resulting in 0-100% virus-caused mortality among 24 treated fifth instars, and 70-100% virus-caused larval mortality among 76 treated sixth instars. No virus-caused mortality was evident among 24 control fifth instars (males) and 33 control sixth instars (females).

To follow the development of infection in larvae, subsequent to ingestion of NPV, abdomens were excised from sacrificed insects or hemolymph samples were taken from a

clipped proleg or cremaster of living insects, then DNA purified from these abdominal tissues or hemolymph was probed for the presence of viral DNA. NPV-treated and control larvae were randomly assigned to one of three subgroups and were subsequently handled in one of three ways: (1) larvae were sacrificed as ultimate instars at 13 days (early dosed) or 5-7 days (late-dosed) post-treatment by freezing, (2) hemolymph was collected by clipping a proleg after an interval ranging from 4-16 days (mean = 11.5 days) post-treatment for early-dosed larvae or 3-7 days (mean = 6 days) post-treatment for late-dosed larvae, or (3) larvae were not sampled but were allowed to die or to survive to the pupal stage. Lethal time and cause of death was recorded for each individual; death was attributed to NPV if PIB were observed upon microscopic examination of cadavers. Virus-treated and control animals that survived to the pupal stage were handled similarly: (1) were sacrificed at 4 to 6 days post-pupation; (2) hemolymph was collected by clipping the cremaster at 12-24 hr post-pupation; or (3) pupae were not sampled but were allowed to die or to eclose to the adult stage. Sacrificed larvae and pupae, and insects that died during the pupal stage or that survived to the adult stage were held in plastic containers at -20^{o}C until ready for tissue masceration and/or DNA purification. Larval and pupal hemolymph samples were collected into sterile 1.5-ml polypropylene micro-centrifuge tubes containing a few crystals of phenyl thiourea to

prevent melanization, and were stored at -20°C until used for DNA extraction and purification. The volume of hemolymph obtained from each larva and pupa ranged from ca. 10-500 ul with a mean of 52 ul from early-dosed larvae and 131 ul from late-dosed larvae, and 65 ul from pupae.

To prepare larvae, pupae and adults for DNA purification, the terminal three to four abdominal segments of each insect were cut off with sterile scissors and mascerated using a plastic pestle (Kontes, Vineland, New Jersey), in 300 ul of buffer (100 mM Tris/HCl 100mM EDTA pH 7.4) in a 1.5-ml polypropylene micro-centrifuge tube. Mascerated samples were kept on ice or were frozen until ready for the DNA extraction and purification procedure.

Mascerated tissue and hemolymph samples were incubated with 0.1 volumes of 10% sodium dodecyl sulfate and 0.018 volumes of proteinase-K (20 mg per ul) overnight at 37°C to denature proteins. DNA was then purified by phenol extraction and ethanol precipitation of DNA (Sambrook et al. 1989). DNA samples were quantified by fluorimetric assay with Hoechst dye (Labarca and Paigen 1980). Each DNA sample was diluted with sterile distilled water to 0.1 ug, 0.2 ug, 0.5 ug, 1 ug, or 2 ug (the maximum amount of DNA possible, depending on the original concentration of purified DNA obtained from each sample) to a final volume of 180 ul. Samples were vacuum blotted onto nitrocellulose filters (BA85, Schleicher and Schuell, Keene, New Hampshire) soaked in 1.0 M ammonium acetate, by a modified slot-blot procedure

(Kafatos et al. 1979), using a Minifold II vacuum slot blotter (Schleicher and Schuell). Filters were baked in a vacuum at 80°C for 2 hr and stored at 4°C in a desiccator until probed.

DNA for labelled probes and DNA standards was purified from virions obtained from PIB using the procedure described by Keating et al. (1989). Covalently-closed, circular viral DNA was labeled by random-primed incorporation of digoxigenin-dUTP (Genius^{R} DNA labeling and detection kit, Boehringer Mannheim, Indianapolis, Indiana) for use as the probe. Hybridization was carried out at 68°C, for 6-12 hr, with agitation. Excess probe was removed at room temperature with two 2X SSC washes, followed by two 0.1X SSC washes.

The kit's detection reagents (alkaline phosphatase-conjugated antibody and substrate) were used to detect probe-hybridized viral DNA in insect samples and the concentration of viral DNA in each sample (ng viral DNA per ng total DNA) was determined by comparison of the density of each DNA blot with densities of standards. The density of each positive sample was measured using a Model SLR-2D/1D soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, California). In addition, each sample was scored as positive for the presence of viral DNA, if the density of the hybridized sample was greater than that of the lowest detectable viral DNA standard. The frequencies of positive and negative samples from each time of treatment (early- or

late-dosed), host stage (larva, pupa, or adult) at time of sampling, sample tissue type (hemolymph or mascerated abdominal tissues), and subsequent fate of unsacrificed animals, were tallied and percentages positive were calculated.

Frequency data were analyzed by chi-square tests of association using SAS (SAS Institute, Cary, North Carolina) PROC FREQ. Fisher's exact test was used to test for associations involving small expected frequencies (SAS Inst. 1987). Individual chi-square statistics for pairwise comparisons among multiple-level variables were calculated according to Steel and Torrie (1980). The effects of time of treatment, larval mortality level, tissue type, sample stage, and subsequent host fate, on the concentration of viral DNA detected in samples were analyzed by ANOVA using SAS PROC GLM.

Results

Concentrations of Viral DNA Detected

As little as 1 ng of viral DNA was consistently detectable, and as little as 10 pg viral DNA was detected in some hybridizations. Among those samples in which some viral DNA was detected, the concentrations (Table 3.1) varied among samples and was not consistently related to

host stage (P = 0.99), tissue type (P = 0.64) or larval mortality category (P = 0.42). However, viral DNA concentration was significantly affected by the time of treatment (P = 0.05). Tissues taken from late-dosed insects had more virus DNA than did early-dosed insects. Based on a median insect cell genome size of 10^6 kb (Lewin 1987) and a virus genome size of 166.6 kb (Smith et al. 1988), the amount of virus DNA detected represents a range of about 50 to 1,320 virions per host cell in pupae and 50 to 2,225 virions per cell in larvae.

Frequency of Detection of Viral DNA in Larvae, Pupae, and Adults

The frequency with which viral DNA was detected among mascerated larval tissue samples was significantly greater than among larval hemolymph samples (P < 0.01) (Table 3.2). The time of treatment did not significantly affect the frequency with which viral DNA was detected among larval tissues (P = 0.542). Viral DNA was not detected in tissues from any of the control larvae.

In pupae, as in larvae, the frequency of viral DNA detection was greater among mascerated tissue samples than among hemolymph samples (P = 0.012) (Table 3.2). The frequency of viral DNA detection among pupal samples was not associated with larval mortality level (P = 0.184). The

time of treatment did not have a significant effect on the frequency of viral DNA detection among pupal hemolymph samples (P = 0.605), however, among mascerated pupal tissue samples viral DNA was detected significantly more frequently among samples from late-dosed insects than those from early-dosed insects (P = 0.001). Viral DNA was not detected in any mascerated adult tissues.

Development of NPV Infection During Metamorphosis

Although less DNA was extractable from hemolymph, and the rate of viral DNA detection in hemolymph was smaller, compared with that obtained from mascerated tissues (Table 3.2), the advantage associated with the use of hemolymph samples is that it enabled me to monitor the progression of viral infection prior, during, and subsequent to metamorphosis, without sacrificing the insect, thus allowing me to determine its eventual fate. The mortality rate was higher among pupae from which hemolymph was obtained (39.7% for NPV-treated and 42.3% for control pupae) compared with unbled pupae (22.0% and 22.2% for treated and control pupae, respectively), however this affect did not appear to bias the results, as the mortality rates were the same for both NPV-dosed and control groups (P = 0.849).

Among larvae in which NPV was detected in the hemolymph, only one survived to the pupal stage. That

individual died two days after pupation. Viral DNA was detected in pupae less frequently than in larvae (Table 3.2). NPV appeared to be a minor cause of pupal mortality, as viral DNA was detected in only one of eight hemolymph samples taken from pupae that eventually died. Among pupal hemolymph and mascerated pupal tissue samples combined, 20% of 71 NPV-dosed insects had viral DNA. Among all individuals surviving to the adult stage, none had detectable viral DNA in hemolymph extracted from them during immature stages.

Discussion

It has often been assumed that NPVs are vertically transmitted within eggs produced by infected adults. Direct evidence in support of this idea is lacking, however, indirect evidence has been cited in support of vertical transmission of NPV in some Lepidoptera. NPV has been detected in tissues of progeny of NPV-infected Spodoptera frugiperda (J. Fuxa, personal communication). Shapiro and Robertson (1987) and Doane (1967) reported detection of NPV PIBs in adult gypsy moth tissues by phase-contrast microscopy, and suggested that NPV-infected female adult moths may transmit inoculum to eggs. However, light microscopic detection of PIB in adults may not be a reliable indicator of productive infection, as PIB bereft of virions

have been observed to predominate in some adult S. frugiperda (Fuxa, personal communication). In fact, other data suggest that lepidopteran pupal and adult tissues may be largely resistant to NPV, due to 'maturation resistance'. Such resistance, or decreasing susceptibility to NPV with advancing developmental age, has been described for several of the Lepidoptera (Evans 1983, Vail and Hall 1969, Whitlock 1977). Maturation resistance could involve interruption of NPV infection during metamorphosis as proposed by Stairs (1965).

In this experiment I was unable to detect NPV in adult tissues of insects which survived NPV treatment, or of any untreated insects. NPV was infrequently detected in pupae compared with larval samples. All but one larva in which NPV was detected died prior to pupation and all pupae in which virus was detected died prior to eclosion. These results suggest that some changes occur during late larval development and metamorphosis which result in reduced viral replication rates among pupae and no detectable virus in adults. Reduction in the number of individuals containing detectable levels of viral DNA following gypsy moth pupation is in agreement with a similar finding for T. ni (Vail and Hall 1969) and is consistent with the decrease in production of PIB after pupation observed in infected gypsy moth (Shapiro and Robertson 1987).

Viral DNA was also more prevalent in pupae from the late-dosed group compared with the early-dosed group. Evans

(1983) found similar results for Mamestra brassicae. In my experiment this difference may have been the result of higher doses administered to late-dosed larvae, which were necessary to produce mortality responses similar to those for the early-dosed larvae. Variability in the concentration of viral DNA detected among samples of similar tissue type, host developmental stage, and treatment might be attributable to heterogeneity in viral production rates among individual hosts.

All insects whose hemolymph had detectable virus eventually died, however, upon microscopic examination of cadaver tissues, PIB were not detected in all cases. It is unlikely that these represented falsely positive individuals because I found no false positive samples among the untreated controls. Rather, these results suggest that either PIBs are not produced or that too few PIBs to be detectable by light microscopy are produced in some infected individuals. I was unable to determine if NPV was responsible or contributed to death of these infected larvae. Perhaps at high doses the virus is capable of interfering with normal physiological processes, such as has been shown for Autographa californica NPV infecting Spodoptera frugiperda (O'Reilly and Miller 1989), leading to premature death before there is adequate time for the virus to produce the large numbers of polyhedra normally associated with viremia.

These results indicate that among adult moths surviving a sublethal dosage of NPV, viral replication does not occur, or occurs at a rate below my level of detection of 50 virus copies per host cell. Therefore, transmission of significant amounts of infectious virus to progeny appears unlikely.

My results contrast with those of Shapiro and Robertson (1987) who reported NPV-caused mortality among progeny of NPV-challenged gypsy moth. It is possible that conditions required for transmission of NPV by infected gypsy moths to eggs were not met in our study, or that the different strains of NPV used contributed to our differing results. However, because Shapiro and Robertson reared larvae in groups, through pupation, on NPV-contaminated diet, it is possible that transmission of NPV to progeny in their experiments resulted from external contamination of adults. I attempted to minimize the risk of external contamination of insects by inoculating with a droplet of NPV and rearing larvae individually, which might explain why I did not observe any evidence of vertical transmission.

I can only speculate as to what kinds of mechanisms might lead to reduction in susceptibility during and following host metamorphosis. It has been suggested that maturation resistance could occur when an insect pupates before a full cycle of viral replication can be completed (Evans 1983). Such an effect might explain why I detected virus in few pupae. However, the fact that virus was found

in more late-dosed pupae than early-dosed pupae indicates that some other factors also may play a role. Stairs (1965) demonstrated the controlling effect that host cell metabolism has on viral replication rate and showed that disease development is interrupted during Galleria mellonella pupation (Stairs 1970). Hormonal or other chemical or structural changes that occur during metamorphosis might also affect the development of NPV infection in gypsy moth. Conceivably, such changes could effectively disrupt disease development, possibly due to a dramatic change in cells and tissues from permissive for viral replication to semi- or nonpermissive, thereby resulting in immunity or resistance of adults, as was suggested for H. armigera (Whitlock 1977). Very low levels of viral DNA such as might exist in adults as a result of a switch from productive to persistent infection (Burand et al. 1986) could perhaps be detected in future work with the use of the polymerase chain reaction (PCR) technique.

Alternatively, it is possible that survivors avoided infection entirely via pre-invasion or invasion-level immunity operating at the midgut barrier. Keddie et al. (1989) showed that some Trichoplusia ni larval midgut cells infected with A. californica NPV are sloughed into the midgut lumen. Such a process may serve as a defensive mechanism. Immunity mechanisms which prevent establishment of infection in midgut epithelial cells have been cited as

the primary mechanism of insect defense against baculoviruses (Briese and Podgwaite 1985).

It is clear that metamorphosis substantially affects the progression of NPV infection in the gypsy moth, as has been demonstrated for G. mellonella (Stairs 1965) and M. brassicae (Evans 1983). I failed to show any evidence of sublethal infection among insects surviving to the adult stage. These results suggest that even among insects that ingest high doses of NPV, those that survive to the adult stage might not harbor virus, and thus, transovarial transmission by NPV-dosed gypsy moths might not occur, or may be limited to persistent or latent infection.

Table 3.1. Concentration of viral DNA in viral DNA-containing hemolymph and mascerated abdominal tissue samples from early-dosed and late-dosed gypsy moth larvae and pupae.

Host Stage Sampled	Tissue Type	Time of Treatment	N	Viral DNA Concentration (ng viral DNA/ng total DNA) range	mean	SE
Larvae	hemolymph	early-dosed	15	0.0008 - 0.0422	0.0118[a]	0.0033
		late-dosed	6	0.0040 - 0.2225	0.0490	0.0348
	mascerated abdomens	early-dosed	8	0.0060 - 0.0663	0.0293	0.0089
		late-dosed	7	0.0188 - 0.1638	0.0663	0.0196
Pupae	hemolymph	early-dosed	2	0.0325 - 0.0359	0.0340	0.0017
		late-dosed	1	0.0285 - 0.0285	0.0285	--
	mascerated abdomens	early-dosed	3	0.00002-0.00004	0.00003	5.9×10^{-6}
		late-dosed	8	0.0114 - 0.1320	0.0619	0.0141

[a]Overall, mean concentrations of NPV DNA were significantly lower ($P = 0.05$) in early-dosed insects compared with late-dosed insects.

Table 3.2. Percentage of viral DNA-containing hemolymph and mascerated abdominal tissue samples from early-dosed, late-dosed, and untreated gypsy moth larvae, pupae, and adults, presented for low (0-25%), medium (26-69%), high (70-93%) and extreme (100%) larval mortality categories.

	Larvae				Pupae				Adult	
Dosage Ingested	Hemolymph		Abdomen		Hemolymph		Abdomen		Abdomen	
	%	(N)	%	(N)	%	(N)	%	(N)	%	(N)
Untreated	0	(37)	0	(6)	0	(22)	0	(22)	0	(17)
Treated - Early-dosed										
Mortality Category										
Low	4.6[a]	(43)	-	(0)	5.7	(35)	10.8	(37)	0	(37)
Medium	43.0	(23)	100.0	(7)	0	(4)	12.5	(16)	0	(7)
High	20.0	(15)	-	(0)	9.0	(11)	-	(0)	-	(0)
Treated - Late-dosed										
Low	20.0	(5)	100.0	(3)	0	(1)	100.0	(3)	-	(0)
High	20.0	(15)	66.7	(3)	12.5	(8)	25.0	(8)	-	(0)
Extreme	25.0	(8)	100.0	(2)	0	(3)	60.0	(5)	-	(0)

[a]In overall comparison, the percentages of hemolymph samples containing detectable NPV DNA were significantly smaller ($P \leq 0.01$) than percentages of NPV DNA-containing mascerated abdominal tissue samples.

CHAPTER 4

EFFECT OF POPULATION QUALITY ON GYPSY MOTH LARVAL SUSCEPTIBILITY TO NUCLEAR POLYHEDROSIS VIRUS

Introduction

One factor which may interfere with effective utilization of NPVs as microbial control agents is that susceptibility to these pathogens can vary widely among different populations of the same insect species (Martignoni and Schmid 1961) or between different years within the same population (Bird and Elgee 1957). Geographically distinct populations of gypsy moth may differ in response to the same strain of NPV by over 100-fold (Rollinson and Lewis 1973). These differences could be due to environmental factors. For example, temperature (Thompson 1959), light (Smirnoff 1967), diet (Keating and Yendol 1987), and exposure to chemicals (Ignoffo and Montoya 1966) have all been shown to affect insect susceptibility to NPV.

Variation in insect susceptibility has also been shown to have a genetic component (Briese et al. 1980, Reichelderfer and Benton 1974, Fuxa et al. 1988). Myers (1988, 1990) speculated that genetic selection for resistance to NPV contributes to cyclic fluctuations in insect population density. Vasiljevic (1961) examined the relationship between changes in population density and

susceptibility and concluded that gypsy moth susceptibility to NPV increases during an outbreak phase and decreases in declining and endemic phases. However, it is generally accepted that the prevalence of NPV in gypsy moth populations increases with population density (Campbell 1963, Doane 1976a,b). There is not a clear indication that environmental sources of inoculum were eliminated in Vasiljevic's study, therefore, increased inoculum levels might account for the increased infection rates observed in dense populations. However, as discussed by Elkinton et al. (1990), higher levels of inoculum present in dense populations could also lead to genetic selection for resistant strains.

Although changes in susceptibility to NPV associated with variation in insect population density have not been conclusively demonstrated for the gypsy moth, several physical, physiological, developmental, and behavioral changes associated with population density fluctuations have been noted. These qualitative changes, which include alterations in feeding rhythms (Lance et al. 1986, 1987), hemolymph carbohydrate concentrations (Greenblatt and Barbosa 1980), and development rate, pupal weight and fecundity (Campbell 1978, Leonard 1970b, Valentine et al. 1983, Wallner and Walton 1979), or their underlying mechanisms, might also affect the susceptibility of gypsy moth to NPV.

I hypothesized that changes in population quality, or in the prevalence of NPV, associated with fluctuations in population density, affect larval susceptibility to the virus. In order to test this hypothesis, I challenged larvae from ten different populations differing in density, phase of outbreak cycle, and NPV inoculum levels, over a two year period with NPV and related their responses to population density, measures of population quality, and prevalence of NPV.

Materials and Methods

Gypsy Moth Populations

Ten populations located in the Eastern Shore region of Maryland were selected for study. All ten populations were examined during the first year (1988), however, one population (Price) was sprayed after the first year and therefore, was excluded from the study in 1989. For each population, density was estimated by counting the number of egg masses found within one or more circular plots. The size and number of plots in which egg masses were counted, and the time of year (fall or spring) in which counts were done (Table 4.1), varied among populations due to different protocols employed by the several agencies assisting in the study. Egg masses from each population were collected in

March 1988 and 1989 by scraping each one into a sealable plastic bag or a 30-ml covered container. Eggs were held at 10°C until June of the year in which they were collected.

Egg mass size, which has been shown to be negatively correlated with population density (Campbell 1978), was determined by measuring the maximum length, to the nearest mm, of 30 randomly selected egg masses from each population. In addition, 10 randomly selected egg masses from each population were weighed to the nearest mg, and the total number of larvae hatching from each of 10 egg masses per population was also counted.

To obtain a measure of the relative prevalence of NPV in each population at the beginning of the larval period, the amount of NPV inoculum associated with egg masses (egg mass virus load) was determined by individually hatching 20-30 untreated egg masses from each population, in clean 30-ml covered plastic containers or in sterile plastic petri dishes. Twenty newly hatched, randomly selected larvae were transferred from each egg mass into an 180-ml cup containing 85 ml of artificial diet (1988) (Bell et al. 1981) or into 5 30-ml cups (4 larvae per cup) containing 15 ml of diet (1989) and were reared at 27°C ($\pm$ 1°C), ca. 50% RH. The photoperiod was 16:8 (L:D). After 10 days, the number of larvae from each egg mass which died due to NPV infection was counted.

Bioassays

Prior to hatch, egg masses from each population were surface-disinfected by soaking them for 1 hr in 3.5% formaldehyde, rinsing with water for 1 hr, patting dry with paper towels, and allowing them to air dry for ca. 3 hrs, after which, each was placed individually into a covered sterile plastic petri dish. Eggs were allowed to hatch at 28°C. A suspension of NPV was fed to 24- to 48-hr old larvae by a droplet-feeding bioassay method (Hughes et al. 1986). Control groups imbibed droplets of distilled water only.

Wild isolates of NPV contain a heterogeneous mixture of genotypes (Shapiro et al. 1984, Vasiljevic and Injac 1973) which could cause variability in susceptibility to infection and mortality. In order to eliminate this source of variability, I used plaque-purified isolate Ld-g (Smith et al. 1988) passed through one generation of New Jersey Strain larvae. Viral PIBs were purified from larval cadavers according to the procedure described by Keating et al. (1989). The concentration of a stock suspension of PIB was determined by counting the number of PIBs in an aliquot using a hemocytometer. Serial dilutions of 1.2×10^4, 1.2×10^3, and 1.2×10^2 PIBs per ml of distilled water were administered in 1988, corresponding to approximate LD_{10}, LD_{50}, and LD_{90} dosages. In 1989, dosages of 1×10^5, 2×10^4, 4×10^3, and 8×10^2 PIBs per ml were administered,

corresponding to approximate LD_{10}, LD_{40}, LD_{75}, and LD_{100} dosages.

To confirm ingestion of the virus suspension, 0.5% (w/v) blue food coloring (FD&C Blue No. 1, Warner Jenkinson, St. Louis, Missouri) was added to the virus suspension (van Beek and Hughes 1986), which was visible through the lightly pigmented cuticle of first instars when they were held up to a light source. In order to estimate the volume of virus solution (and hence, the number of PIBs) ingested, an additional group of larvae was fed a solution of blue coloring (0.5 mg/ml) and sodium fluorescein (2.4 mg/ml) in distilled water as described by van Beek and Hughes (1986). Immediately after ingestion of the fluorescein solution, each larva was crushed with a glass rod in a test tube containing 3 ml sodium phosphate buffer (0.05 M, pH 7.7). After vortexing, the volume ingested by each fluorescein-fed larva was calculated by determining the fluorescence of each sample with a fluorescence spectrophotometer set at an excitation wavelength of 481 nm and an emission wavelength of 512 nm. Calibration curves (regression of logarithm of fluorescence against logarithm of concentration of sodium fluorescein) were established for each test using half-fold dilutions of fluorescein ranging from 240 to 15 ng/ml buffer.

In 1988, larvae hatching from each egg mass were treated independently from all other egg masses. From each egg mass, approximately 100 larvae were randomly assigned to

one of five treatments. Three concentrations of NPV, the control solution, and the florescein solution were each administered to a group of approximately 20 neonates from each egg mass. Eight to twelve egg masses per population were treated in this manner, for a total of 480-720 NPV-dosed, 160-240 control, and 160-240 fluorescein-fed larvae from each of ten populations. Larvae were reared individually in 30-ml covered plastic cups containing 15 ml of artificial diet. Larvae were reared at 27°C 16:8 hr (L:D) and ca. 50% RH for 10 days, and were checked daily for mortality.

Bioassays conducted the second year (1989) followed a similar protocol except for the following changes. Larvae hatching from several egg masses were pooled, within each population, for treatment. Approximately 150 larvae per population were randomly assigned to one of six treatments (25 larvae per treatment): four concentrations of NPV, the control solution, and the fluorescein solution. This was replicated three to five times for a total of 300-500 NPV-dosed larvae, 75-125 control larvae, and 75-125 fluorescein-fed larvae from each population. Dosed and control larvae were reared in groups of 5 in 30-ml cups and mortality was assessed after 10 days. One population (C.Hill) was inadvertantly excluded from the 1989 bioassays. Time to response (LT_{50}) was not measured the second year.

Data were analyzed with SAS computer programs (SAS Institute 1987). The standard curve for the fluorescein

solution used to estimate the mean volume of solution ingested by larvae was fit by PROC REG and the mean volume ingested was calculated for each population and replicate using PROC MEANS. The concentrations of NPV administered were adjusted for the estimated volumes ingested for each group to yield an approximate dosage ingested by each NPV-dosed group. Regression parameters for logistic regressions of the ingested dosage against response (mortality) and dosage administered against time-to-response were obtained using the SAS program for categorical data (PROC CATMOD). The variances of the LD_{50} and the LT_{50} for each population were calculated using a formula for ratios (Miller 1986). Differences in LD_{50} among years were tested independently for each population with PROC TTEST. Correlations among susceptibility, measures associated with population density (egg mass counts and egg mass measurements), and the amount of NPV inoculum associated with egg masses were obtained by PROC CORR. Population, replicate, and year effects on LD_{50} and dosage, population, and replicate effects on LT_{50} were tested by ANOVA, weighted by the inverse of the variance to stabilize unequal variances, using PROC GLM. The effects of population density, egg mass size, and egg mass NPV-contamination (egg mass virus load) on susceptibility were examined by weighted analysis of variance (PROC GLM) and were modelled by stepwise regression (PROC REG). Models were limited to two independent variables because models

with a greater number of variables appeared to be overspecified as evidenced by their extremely high R^2.

Results

The dosage-mortality regression parameters for each of the ten populations tested in 1988 and eight populations tested in 1989 are shown in Table 4.2. Mean egg mass measurements and the estimates of egg mass virus load (percentage NPV-caused mortality among first instars hatching from untreated egg masses) for each population are shown in Table 4.3.

Models which best describe (highest R^2) the contribution of population measures (means of egg mass length, egg mass weight, number of larvae hatching per egg mass, egg mass virus load, and population density) to the variability in mean LD_{50} for both years combined and for 1989 alone are shown in Table 4.4. When data from both years were pooled, the year of the study had the biggest effect. Four out the eight populations for which complete data were obtained (B, E, Ch2, and S) showed significantly ($P \leq 0.05$) lower susceptibility (higher LD_{50}) in 1989 compared with 1988. Two additional populations (C and 2) were significantly less susceptible in 1989 at the $P = 0.06$ level. Egg mass virus load also contributed to the combined-years model ($P = 0.07$), with a positive regression

parameter indicating that populations with egg masses carrying greater amounts of NPV inoculum had higher LD_{50}s, thus, were less susceptible to NPV than were populations with egg masses with smaller virus inoculum loads.

Slopes of the dosage-response curves did not vary significantly among populations (P = 0.44), therefore, within-population variability did not differ greatly among the populations studied. However, slopes varied significantly with year (P = 0.01) indicating that variability within the populations changed from 1988 to 1989.

When each year's results were examined independently, there were significant differences in LD_{50} among populations within each year (P = 0.04). In 1989, egg mass virus load had a significant effect (P = 0.03) if egg length was also included in the model, although the latter factor did not contribute significantly (P = 0.12). Egg mass virus load was low (less than 10%) in all populations in 1988 and did not contribute significantly to variability among LD_{50}s in that year. In fact, variability among populations in 1988 was unexplained, as none of the population measures contributed significantly.

No significant differences in the time to mortality (LT_{50}) were detected among the ten populations challenged in 1988 (P = 0.20), therefore this parameter was not examined in 1989. Higher dosages administered resulted in slightly, but significantly faster mortality (P = 0.0001) (Table 4.5).

The effect of dosage on LT_{50} did not vary among populations (P= 0.57).

Discussion

I hypothesized that fluctuations in population density affect susceptibility of gypsy moth larvae to NPV. All populations appeared to be increasing numerically in 1988, as estimated by egg mass counts, however, in 1989, three populations continued to increase (A, E, and C.Hill), four populations declined (D, C, 2, and S), and one remained relatively stable (Ch2). Although many of the population density estimates were based on a small number of samples, even one 0.01 ha plot should indicate whether the populations were increasing or declining given the extreme density fluctuations evident in these populations. I did not find a clear relationship between phase of outbreak cycle or population density and LD_{50}.

Although no direct relationship between population density and susceptibility was evident, egg mass virus load, a factor associated with population density and NPV prevalence in gypsy moth populations (Doane 1971, 1976a, 1976b, Woods et al. 1990), accounted for some of the variability observed. Increased prevalence of NPV is generally correlated with increased population density for gypsy moth (Campbell 1963, Doane 1976a, 1976b), as well as

for other insects (Evans 1986, Anderson and May 1981). Such a relationship was evident in this study also; egg mass virus load in 1988 was significantly correlated with population density the previous year (P = 0.04) and in the same year (P= 0.04). Because egg mass virus load was found to explain some of the variability in susceptibility (Table 4.4), these results suggest that changes in population density may contribute, at least indirectly, to the differences in susceptibility observed among gypsy moth populations.

Because egg mass inoculum is primarily acquired from previously-contaminated substrates (Chapters 1 and 2), and therefore, reflects the relative prevalence of NPV in the previous generation, it is possible that genetic selection for resistance to NPV contributed to the significant increases in LD_{50} evident among six of the eight populations which were studied in both years. This is supported by my finding that the population that showed the greatest increase in resistance (population S) also had the highest egg mass inoculum level.

Martignoni and Schmid (1961) hypothesized that epizootics select for resistance to pathogens by removing susceptible individuals from the population. In fact, artificial selection for resistance to NPV has been demonstrated in laboratory populations (Fuxa et al. 1988). However, Briese (1986) distinguishes between 'true resistance' and resistance arising from removal of

susceptible individuals. The former, characterized by a shift in mean response with no change in the slope of the dose-response regression, indicates an increase in the frequency of resistance genes, whereas, the latter results in reduced variability in response (increased slope) but does not result in changes in the frequency of resistant alleles. Because increases in the mean LD_{50}s in 1989 were accompanied by decreased, rather than increased slopes, this decreasing susceptibility could represent the development of true resistance in most of the populations the second year.

Alternatively, factors other than genetic selection for resistance may also have contributed to increased variability observed in 1989, relative to that observed in 1988. One possibility is that dispersal of individuals from and to adjacent populations resulted in random genetic mixing rather than directed genetic selection. Air-borne 'ballooning' of first instars is the most important means of dispersal (Leonard 1971), therefore, larval dispersal could have contributed to a shift in mean susceptibility as well as increased variability observed in some populations in 1989 relative to the previous year.

Another possibility is that the increased variability resulted from changes in the bioassay procedure during the second year of the study. Each family (progeny of a single female moth) was tested independently in 1988, while in 1989, larvae hatching from three to five egg masses were pooled within each population for each replicate.

Therefore, genetic variability among families, which was distinguishable from among-replicate variability in 1988, but indistinguishable in 1989, may have contributed to increased variability in response (smaller slope) seen in 1989 relative to 1988. However, a genetic or environmental effect, rather than one due to bioassay technique, may be supported by the significant population by year interaction ($P = 0.05$), indicating that the differences among years were not constant among the different populations.

It is perhaps important to consider that factors other than those affecting the susceptibility of first instars could also have an impact on the expression of the pathogen in natural populations. For instance, different strains of NPV differ in virulence (Shapiro et al. 1984, Vasiljevic and Injac 1973). This source of variation was eliminated in the experiment with the use of a genetically pure strain of NPV and removal of natural sources of inoculum by surface disinfection of eggs. However, in natural populations, variability in virulence could counter or accentuate differences in insect susceptibility. Also, insect susceptibility could be viral strain-specific; an insect or population which is susceptible to one virus strain could be resistant to another. The effects that population quality, density, or the prevalence or virulence of NPV have on the susceptibility of neonates to the virus could also be overwhelmed by other environmental factors affecting the expression of the pathogen among later instars. It is

probable that the effect of varying egg mass virus inoculum level is just one of a number of factors which interact to determine population susceptibility to NPV. However, because prevalence of the disease among neonates is the major source of inoculum available for secondary infections (Doane 1969, 1970, Woods and Elkinton 1987), factors influencing susceptibility of neonates are likely to play an important role in the generational impact of the disease.

Although more research is needed in order to fully define the role that population dynamics play in determining susceptibility to NPV, these results indicate that prevalence of NPV, which is known to change with fluctuations in density, may influence gypsy moth larval susceptibility to this pathogen.

Table 4.1. Population densities of the ten study populations from the Eastern Shore region of Maryland.

Population	Estimated Density (egg masses/ha)[1] 1987	1988	1989
A	0	3213	46306
B	0	2422	nc[a]
C	4060[2]	46717[2]	20490[3]
D	2913[2]	70117[2]	5140[3]
E	0	1235	27823
2	892[2]	10583[2]	2501[3]
Ch2	0	1828	1631[3]
C.Hill	0	3805	13590[3]
S	1799[2]	42909[2]	8135[3]
Price	0	11785	nc

[1]estimate based on the number of egg masses counted within 1 0.1-ha circular plot.

[a]nc = not counted.

[2]estimate based on the number of egg masses counted within 15 0.1-ha circular plots.

[3]estimate based on the number of egg masses counted within 3 0.1-ha circular plots.

Table 4.2. Parameters of mean log dosage-mortality response for each of ten Maryland gypsy moth populations collected in 1988 and 1989 and challenged with NPV the same years in laboratory bioassays.

Population	N	Log LD_{50} Mean	SE	LD_{50} (PIBs)[1]	95% Fiducial Limits[2]	Slope Mean	SE	Intercept Mean	SE
1988									
A	11	1.45	0.23	28.1	15.2-151.7	2.69	0.79	-4.58	1.98
B	9	1.12	0.16	13.2	3.9- 44.4	3.87	1.15	-4.37	1.31
C	10	1.30	0.16	20.0	5.9- 67.3	7.07	1.49	-8.87	2.19
D	10	1.65	0.17	44.7	12.8-155.7	2.48	0.53	-4.03	0.91
E	12	1.38	0.14	23.8	8.7- 66.4	3.78	0.82	-5.01	1.15
2	10	1.31	0.12	20.4	8.2- 50.9	3.14	0.80	-4.80	1.80
Ch2	8	1.16	0.15	14.4	4.4- 47.1	3.76	1.20	-4.23	1.42
C.Hill	9	1.11	0.20	13.0	2.8- 59.1	5.20	1.40	-6.65	1.98
S	10	1.07	0.16	11.7	3.4- 40.2	3.15	0.32	-3.60	0.90
Price	10	1.52	0.18	33.0	8.2-132.5	4.03	0.98	-6.13	1.92
1989									
A	3	1.61	0.13	41.0	2.1-815.2	2.49	0.87	-4.27	1.38
B	5	1.75	0.11	56.5	18.0-177.2	2.44	0.48	-4.36	0.90
C	5	1.80	0.11	62.7	24.1-163.2	2.75	0.15	-4.94	0.33
D	4	1.21	0.11	52.4	11.9-230.8	2.74	0.13	-4.70	0.33
E	5	1.75	0.06	56.5	30.4-104.9	2.54	0.06	-4.46	0.22
2	5	1.69	0.08	48.9	20.1-118.9	2.57	0.23	-4.30	0.31
Ch2	5	1.77	0.09	58.9	22.0-157.6	3.00	0.38	-5.27	0.41
C.Hill	-	-	-	-	-	-	-	-	-
S	4	1.87	0.09	73.5	21.4-253.0	2.17	0.56	-3.66	1.45
Price	-	-	-	-	-	-	-	-	-

[1] antilogarithm of the logarithm mean LD_{50}

[2] 95% fiducial limits calculated as mean $LD_{50} \pm t_{0.005,n-1}$ $(\text{variance}/n)^{-1/2}$

Table 4.3. Means of egg mass length and weight, number of first instars hatched per egg mass, and percentage of untreated first instars (L1) which died due to NPV infection upon hatching from egg masses collected from ten Maryland populations.

Population	length (mm) mean (SE)	weight (g) mean (SE)	no. larvae per egg mass mean (SE)	% L1 NPV mortality mean (SE)
1988				
A	28 (1.1)	0.31 (0.03)[1]	48 (5.4)	0.5 (0.3)
B	32 (1.3)	0.43 (0.03)	504 (54.5)	--
C	24 (1.4)	0.11 (0.03)	152 (15.9)	7.8 (2.5)
D	24 (0.8)	0.21 (0.04)	145 (14.1)	6.4 (1.7)
E	36 (1.5)	1.31 (0.28)	70 (6.5)	2.2 (1.7)
2	28 (0.9)	0.31 (0.02)	203 (18.1)	5.9 (3.6)
Ch2	34 (1.3)	0.40 (0.05)	57 (4.8)	0 (0)
C.Hill			57 (4.5)	5.3 (3.5)
S	31 (1.5)	0.15 (0.02)	344 (25.8)	9.4 (3.7)
Price	30 (1.04)	0.45 (0.03)	47 (7.2)	5.0 (2.7)
1989				
A	26 (0.6)	0.05 (0.005)[2]	125 (22)	6.4 (2.0)
B	24 (1.0)	0.04 (0.005)	124 (18)	4.2 (1.5)
C	25 (1.0)	0.05 (0.006)	145 (15)	23.2 (6.7)
D	22 (1.2)	0.04 (0.005)	67 (11)	8.5 (3.0)
E	32 (1.0)	0.06 (0.013)	208 (29)	2.8 (1.2)
2	23 (1.4)	0.05 (0.004)	109 (25)	14.9 (5.0)
Ch2	28 (1.2)	0.06 (0.004)	204 (30)	8.2 (4.0)
C.Hill	32 (1.2)	0.10 (0.025)	--	--)
S	20 (1.2)	0.04 (0.010)	113 (18)	41.8 (7.0)
Price	--	--	--	--

[1] measured before egg hatch

[2] measured after egg hatch

Table 4.4. Best regression models, and ANOVA tables describing the effects of year, egg mass length, and egg mass virus load on the mean NPV susceptibility (LD_{50}) of first instar gypsy moth larvae hatched from egg masses collected from each of ten Maryland populations. None of the variables tested were significantly related to the LD_{50} in 1988.

Both Years Combined:

Model LD_{50} = -33.6 + 0.40(year) + 0.003(egg virus load)

*R^2 = 0.77 P = 0.0001

Variable	DF	SS	F	P
Year	1	4.16	36.88	0.0001
Egg Mass Virus Load	1	0.42	3.75	0.0733

1989 only:

Model LD_{50} = 1.32 + 0.01(egg length) + 0.006(egg virus load)

*R^2 = 0.59 P = 0.07

Variable	DF	SS	F	P
Egg Length	1	0.25	3.81	0.1226
Egg Mass Virus Load	1	0.65	10.01	0.0340

* adjusted for degrees of freedom.

Table 4.5. Mean LT_{50} (all populations pooled) for mortality among larvae challenged with three concentrations of NPV.

Dosage (PIB/insect)	Number of Replicates	LT_{50} (days) Mean	SE
120	68	6.32a[1]	0.072
1200	104	6.28b	0.047
12000	102	6.06c	0.034

[1]Means followed by the same letter are not significantly different (P=0.05), Tukey's studentized range test.

CHAPTER 5

VERTICAL DISTRIBUTION OF NUCLEAR POLYHEDROSIS VIRUS-INFECTED GYPSY MOTH LARVAE AND EFFECTS ON SAMPLING FOR ESTIMATION OF DISEASE PREVALENCE

Introduction

In addition to the physiological effects that infectious pathogens have on their insect hosts, disease-related behavioral abnormalities have also been described for a number of insect/pathogen associations. For instance, sawflies which normally feed gregariously may switch to solitary feeding habits, or may wander away from a food source when infected with some viruses (Smirnoff 1965). It is a characteristic of many insects infected with Entomophthorales fungi to climb to the top of the host plant (Carruthers and Soper 1987, Entwistle and Evans 1987). A similar behavioral effect termed 'Wipfelkrankheit' or 'tree-top disease' has also been described for a gypsy moth conspecific, the nun moth, Lymantria monacha, when infected with NPV (Komarek and Breindl 1924).

Obviously, such behavioral effects could lead to altered dispersion of infected individuals in a population. In order to estimate the prevalence of disease in a population to aid in pest suppression and pest management decision-making, or to monitor and evaluate the efficacy of

microbial insecticides, a sampling scheme based on the dispersion of infected and healthy individuals in a population, must be developed. However, the effects that NPV infection have on behavior and dispersion of gypsy moth larvae have not been previously examined and sampling methods for estimating disease prevalence in gypsy moth populations have not been standardized.

Collection and rearing of insects with subsequent monitoring for causes and rates of mortality is a method which has been used to estimate pathogen levels in a population (Woods et al. 1987, 1990). Unfortunately, it is often difficult to locate sufficient numbers of gypsy moth larvae in a forest in low and even moderate-density populations, particularly during late larval stages when larvae spend daylight hours resting in cryptic places (Forbush and Fernald 1896). Burlap bands wrapped around tree boles provide artificial resting sites highly favored by late instars, and have been used to facilitate collection of larvae for pest control (Forbush and Fernald 1896) and for detecting and monitoring gypsy moth populations (McManus et al. 1980, McManus and Smith 1984).

Burlap bands have also been used for estimating larval mortality rates due to various causes, including NPV (Webb et al. 1989, 1990). Although, the use of burlap bands in this manner greatly simplifies sampling, it is not known if sampling from burlap bands alone provides an unbiased estimate of the impact of NPV on a population. To develop a

sampling protocol that provides an unbiased estimation of disease prevalence, the dispersion of infected individuals within a population must be known.

This study was undertaken to determine the vertical distribution of NPV-infected gypsy moth larvae in forested sites and to evaluate the use of burlap bands for estimating the incidence of NPV in gypsy moth populations.

Materials and Methods

I observed the movement and vertical distribution of gypsy moth larvae to determine whether dispersion of larvae is altered by NPV infection, and whether NPV-infected and healthy larvae use burlap bands similarly, as daytime resting sites. In addition, I examined two methods for using burlap bands to estimate disease prevalence: (1) counting cadavers found under burlap bands and (2) rearing larvae collected from under burlap bands. Estimates obtained by these two methods were compared with each other and with the prevalence estimated by rearing larvae collected extensively from the same sites.

Behavioral Observations of Infected and Uninfected Larvae

Behavioral observations of infected and uninfected larvae were done to determine experimentally if NPV infection induces changes in larval movements which could result in altered vertical distribution of infected larvae.

At a site located on Cape Cod, Massachusetts which harbored a very low resident gypsy moth population, scaffolding was erected around a black oak tree, 6 m tall, 12.5 cm DBH. This site was chosen because resident gypsy moth populations had been low for a number of years, therefore the levels of NPV inoculum persisting in the area was also expected to be quite low. Adjacent trees were trimmed so that their branches did not contact the observation tree, and some of the understory vegetation was removed to facilitate movement around the base of the tree. Two burlap bands were wrapped around the bole of the observation tree, at 1 and 1.3 m height. In addition, burlap bands were also wrapped around adjacent trees to aid in the collection of wild larvae and detection of between-tree movement of released larvae.

New Jersey Strain larvae were reared in cups of artificial diet which were held in an outdoor insectary. Within 24 hrs of molting to the third or fourth stadium, larvae were infected with NPV (Gypchek) by feeding each larva a cube of artificial diet (ca 27 mm^3) contaminated with a 10 ul droplet containing ca 10^6 PIBs in sterile

distilled water. Control larvae were fed a cube treated with 10 ul of sterile distilled water. Larvae which failed to consume the entire cube were excluded from the experiment. Virus-fed and control larvae were transferred to separate outdoor cages containing bouquets of black oak foliage held in jars of water. The foliage in these cages was first disinfected by soaking for 30 mins in 0.05% sodium hypochlorite, followed by a 30 min soak in water. Foliage was removed and replaced with fresh foliage every 3-4 days.

Within 48 hrs after molting to the next stadium (fourth or fifth), larvae were collected from the cages and were marked on the dorsum with a spot of fluorescent powder to distinguish the virus-dosed from the control larvae. Larvae from both treatments were released between 0800 and 1600 hrs onto the observation tree by placing them into a paper cup which was attached to the bole of the observation tree underneath one burlap band. Infected larvae died five to ten days after release. As a check, some of the marked larvae from each treatment were not released but were transferred to 28-ml cups containing artificial diet and were kept in an outdoor insectary where they were monitored twice weekly for time and cause of death.

Wild larvae which were collected from the area near the observation tree were also marked uniquely with fluorescent powder and were released along with the infected and uninfected laboratory-strain larvae, to serve as a check for normal movement and activity. Infected, control, and wild

larvae were released in groups of 40 to 80 on each of five different occasions. At the first release, larvae had been infected 6-7 days previous, whereas all subsequent releases were done at 10-11 days post-infection. A total of 121 virus-dosed, 125 control, and 44 wild larvae were released.

Observations of the released larvae were made at periodic intervals after release and at several different times of the day and night. At each observation, the observers systematically searched the entire tree, by climbing on the scaffolding which surrounded the tree on all sides. The location of each larva in the tree, its color marking, and its activity (feeding, resting, walking, or dead) were recorded. Each dead larva was collected into an empty plastic container which was marked with the date and the location on the tree where it was found, and was taken to the laboratory where it was examined microscopically for the presence of PIBs to determine if death was due to virus infection.

Evaluation of Burlap Bands for Estimation of Disease Prevalence.

Study Sites. The study was carried out at three forested sites on Cape Cod, over two consecutive seasons. In 1986, two 9-ha sites were established. Gypsy moth population density was estimated by counting all egg masses

within each of 169 5-m fixed-radius plots (Kolodny-Hirsch 1986) which were arranged in a 13 x 13 grid throughout each site. One site (Otis 1) harbored a high density gypsy moth population (2994 $\pm$ 26 egg masses/ha), and the second site (Otis 5) supported a moderately low density population (311 $\pm$ 10 egg masses/ha). Each site was divided into a 3 X 3 grid of 9 1-ha plots, and each plot was further divided into a 4 X 4 grid of 16 625-m^2 subplots. At the approximate center of each of 3 randomly selected subplots within each of the 9 plots at both sites, burlap bands were stapled around each of 11 trees. Selection of trees was based only on location at the center of the subplot. Tree species was not considered, however those with trunks measuring smaller than 7 cm DBH (trunk diameter at 175 cm above the ground) were excluded.

In 1987, the study was conducted at a third site (DPW) measuring 1.5 ha. Egg masses counted within 15 5-m radius plots indicated that this site supported a very high density gypsy moth population (3574 $\pm$ 265 egg masses/ha). Twenty-five 625-m^2 plots were established within this site; ten burlapped and 15 unburlapped plots alternating in a checkerboard pattern. At the center of each of the subplots chosen for burlap sampling, burlap bands were stapled around the bole of each of 10 trees. As before, the 10 centermost trees were burlapped regardless of species, and trees smaller than 7 cm DBH were excluded.

Larval Collections. To determine the vertical distribution of infected larvae and to compare NPV prevalence among larvae found under burlap bands with that among larvae sampled more extensively, larvae were collected three times at weekly (Otis 5) or biweekly (Otis 1) intervals in the 1986, beginning at peak fourth stadium. Each time, larvae were collected from one burlapped subplot and an adjacent unburlapped subplot within each plot at each site. To avoid the possibility that repeated sampling of the same burlap bands might affect estimates, only one burlapped point within each plot at each site was sampled each time. In this manner, each burlap band and each subplot was sampled only once. Eight randomly selected larvae (4 larvae from each of 2 sides of the tree) were collected from under each burlap band. At the center of each adjacent unburlapped subplot, the understory vegetation, litter, tree trunks, and crowns were searched, using pole-pruners and ladders to search the crowns and upper tree trunks, until 65 larvae were found and collected.

In 1987, larvae were collected each week for 5 weeks, beginning when most larvae were early fourth instars. Every week, 3 randomly chosen larvae were collected from underneath each burlap band within each of the 10 burlapped subplots. Also each week, 10 of the 15 unburlapped subplots were randomly chosen for sampling. At the center of each unburlapped subplot, the tree trunks, large branches, canopy leaves, and litter were searched until ca 4 larvae were

found and collected from each stratum. The approximate height from which each larva was collected was recorded.

Upon collection, each larva was placed individually into a 28-g cup containing 10 ml of artificial diet (Bell et al. 1981), and the lid was marked to identify the location and date of collection. Larvae were held in an outdoor insectary. All larvae collected from under burlap bands and other strata were observed weekly, and the apparent cause and date of mortality were recorded for each larva. Cause of death was attributed to NPV if a large number of PIBs were observed upon microscopic examination of cadaver tissues. Using the method of Woods and Elkinton (1987), weekly NPV-caused mortality within each population was estimated from the percentage of mortality, occurring within ca. one week, among collected larvae. The proportion of larvae collected from under burlap bands which subsequently died within 8 days, due to NPV infection was compared with 8-day percentage of NPV mortality among larvae collected from each stratum and from all other strata combined.

Comparison of Single Versus Repeated Sampling of Burlap Bands. To compare estimates obtained by repeatedly sampling burlap bands with those from sampling each band only once, one burlap band in each subplot was resampled each week. Each time, 8 larvae were collected from each band and reared as described above. Larvae remaining under each band were destructively removed from the tree with a wire brush. NPV-

caused mortality among larvae collected from these repeatedly sampled bands was compared with that among larvae collected at the same time from under previously sampled burlap bands in the same subplots.

Cadaver Counts. In 1986, twelve additional points were established within each site, for evaluating a method of counting the NPV-killed cadavers found under burlap bands as a means of estimating disease prevalence in a population. These points were uniformly distributed throughout each site in a 3 X 4 grid. At each point, burlap bands were stapled around each of 25-35 trees. Counts were done twice in Otis 5 site, at approximately peak fourth, and peak fifth larval stadia. The Otis 1 site was counted once, at peak fourth stadium. The counts were done according to a previously developed sampling protocol (Jones 1985). On each occasion, each burlap band was lifted and the number and stadia of all live gypsy moth larvae found underneath each burlap band were recorded. In addition, the number, stadia, and probable cause of mortality of all dead larvae were recorded. NPV-killed larval cadavers were identified on the basis of gross morphological appearance; flaccid, inverted or inverted-V posture and/or milky-brown liquid oozing from the cadaver.

Cadaver Collections. Because these observations indicated that virus-infected larvae died in the topmost

portions of the tree, several white oak and black oak trees located in site DPW were climbed, to determine if aggregation of NPV-killed larvae at the tree tops was also evident in a natural population. Each larval cadaver found on these trees was collected into a 30-ml container labelled with the height and location in the tree where it was found and was taken to the laboratory where each was examined microscopically for the presence of PIBs. Each cadaver was scored as either virus-killed, if a large number of PIBs were found (average of 10 or more PIBs per microscopic field in three fields), or not virus-killed if few or no PIBs were found.

Data Analyses. Data were analyzed using SAS statistical analysis package (SAS Institute 1987) and chi-square tests. The patterns of virus-caused mortality observed among larvae collected from burlap bands and from all other strata over the three-to-five week periods were compared with the expected mortalities using chi-square tests (Steel and Torrie 1980). The effect of repeated burlap sampling was examined by ANOVA (SAS PROC GLM) of arcsine square root-transformed percentage of mortality among larvae collected from repeatedly and singly sampled burlap bands. The effects of collection height of cadavers, and the effects of height, date, and stratum of live larvae on NPV-mortality rates were also examined by ANOVA (PROC GLM), as was variation in the activity, and height within

the tree of virus-infected and uninfected larvae following their release. Mean separations were determined by Tukey's Studentized Range Test.

Results

Observations of infected laboratory-strain larvae released onto a burlap-banded tree indicated that infected larvae were found significantly less frequently under burlap bands than were healthy larvae ($P < 0.0001$). During daylight hours, infected individuals were found significantly higher ($P = 0.0001$) in the tree than were healthy ones, but at night, both were found primarily in the tree crown (Figure 5.1). Both treated and control laboratory-strain larvae occurred significantly higher ($P < 0.05$) in the tree than the wild larvae released at the same time.

Among these experimentally released larvae it was apparent that infected individuals tended to die at tree top. Most infected larvae died in the top quarter of the tree, and almost half died in the top 30 cm of the crown (Figure 5.2). Mortality among the control group was slight and none died of NPV. Of the few control cadavers recovered, all were found in the lower three-quarters of the tree. Almost all of the cadavers of the wild larvae which were released along with infected and control laboratory-

strain larvae, were found in the lowest quarter of the tree, and none appeared to have died due to NPV infection.

Collection of cadavers from the natural population at site DPW confirmed the observation that NPV-infected larvae tend to die at tree top. Post-mortem dissection of 180 naturally occurring wild cadavers collected from trees, revealed that 39 larvae (21.7%) died due to NPV infection. Among the cadavers of NPV-killed larvae, 56% were found above 4 m, compared with 23% of the cadavers of larvae which died of other causes (Table 5.1).

In the burlap band sampling tests, the percentage of NPV-induced mortality was not significantly different ($P > 0.05$) among larvae collected from burlap bands which were repeatedly sampled compared with mortality among larvae collected from bands which were sampled only once (Table 5.2). This observation was consistent in both sites, therefore, repeatedly and singly sampled bands were pooled for all further data analyses, and the experimental protocol was altered the following year so that all bands were repeatedly sampled.

Comparisons among strata of larval NPV-caused mortality indicated few significant differences (Table 5.3). Only one stratum (bole) in one site (Otis 1) was significantly higher than other strata, although it was not significantly different from burlap mortality ($P > 0.05$). However, when mortality among larvae collected from all other strata within each site was pooled, the pooled mortality was higher

than that among larvae collected from under burlap bands alone (Figure 5.3). This effect was evident in all three sites and in both years, although the disparity between the two sampling methods (burlap versus all other strata combined) was significant ($P < 0.05$) in only two of the three sites (Otis 5 and DPW).

When NPV-caused mortality among larvae was stratified by the height from which larvae were collected, height was not a significant factor ($P = 0.74$). Although the highest mortality occurred among larvae collected from the highest stratum (> 6 m), this was not significantly higher than any other stratum ($P > 0.05$), nor was it higher than the other strata combined ($P = 0.15$).

The results of mortality estimates based on counts of the number of live and dead larvae found under burlap bands in Otis 1 and Otis 5 are also shown in Figure 5.3. Virus mortality, as determined by this method, was significantly lower ($P < 0.05$) in both plots than estimates obtained by rearing larvae collected from all strata.

Discussion

Previous studies indicate some disagreement as to behavioral effects associated with NPV infection in the gypsy moth. Doane (1967) reported partial paralysis, reflected in reduced control of proleg crochet movement,

occurred in infected larvae a few days prior to death, and that feeding ceased before other external symptoms of infection were expressed. Affects on locomotory capabilities were not reported, however, it is quite possible that movement is not seriously affected by such partial paralysis. Lance (1985) found no differences in feeding rhythms of infected and healthy larvae, although he did not indicate how advanced infection was at the time of observation. Liebhold and Elkinton (1986) found no difference in the tendency of larvae that subsequently died from NPV infection to move between trees compared with larvae that did not die from NPV.

Evans and Entwistle (1987) suggested that infection of nerve ganglia and hemocytes mimics starvation, causing insects to continually search for food. This explanation is consistent with our finding that a greater proportion of infected larvae were found in the crown of the tree during daylight hours, compared with uninfected larvae, which more closely followed the normal pattern of seeking cryptic locations on the lower tree trunk or in the litter during the day, moving to the crown to feed primarily after dark. Although I did not find that actual feeding activity was altered in infected individuals, NPV could cause larvae to search for food during the day without stimulating feeding activity. It is apparent that the specific mechanisms causing infected larvae to seek elevated sites are yet to be elucidated.

Both the infected and uninfected laboratory-strain larvae released for observation were found higher in the tree compared with wild larvae released at the same time. To minimize any behavioral effects which might arise due to handling, these wild larvae were collected from burlap bands on adjacent trees just prior to release on the observation tree. Therefore, uncontrollable differences such as in phenological development, genetics, or parasitism or disease among the wild larvae could have contributed to this behavioral difference I observed between laboratory-strain and wild larvae. It is possible that in wild populations, behavioral effects of NPV infection are not as pronounced as that seen among the laboratory-strain larvae, however, the results of the burlap sampling tests and cadaver collections indicate that NPV infection does cause altered vertical distribution of larvae in natural populations.

NPV-caused mortality among reared larvae collected from under burlap bands averaged 78% to 240% lower, and cadaver counts under burlap bands averaged 660% lower than that among reared larvae collected from all strata. Aggregation in the upper canopy and less frequent utilization of burlap bands by infected larvae may explain these effects. I observed that infected larvae were found under burlap bands less frequently and tended to occur higher in the tree during the day compared with healthy larvae. Furthermore, the cadavers of NPV-killed larvae were found primarily at or near the tree top, rather than under burlap bands, which

undoubtedly contributed to the very low estimate of disease prevalence obtained by burlap cadaver counts.

Although larval dispersion is apparently affected by NPV infection, some released and wild infected larvae were found under burlap bands. It is likely that the behavior of infected larvae is not altered until the later stages of infection. Released, infected larvae were never observed in the topmost portions of the crown until after death. Thus, because the time of infection and mortality within a population is not synchronized among all individuals, at any particular time a certain proportion of infected larvae will be in the early stages of infection. Therefore, they are likely to exhibit somewhat normal behavior, including resting diurnally under burlap bands.

Burlap band sampling may, therefore, provide a reasonable estimate of disease prevalence when a high degree of accuracy is not required. Although burlap sampling underestimated the prevalence of the disease in the population, cumulative mortality among burlap samples was roughly proportional to that seen in samples obtained by more extensive larval collection and rearing. Therefore, reasonable estimates could perhaps be obtained from burlap samples by multiplying mortality by some adjustment factor. Our study showed that cumulative NPV-caused mortality among burlap-collected larvae, multiplied by a factor of 1.2 to 2.2, was approximately equivalent to that observed among reared larvae collected from all strata.

Counting cadavers under burlap bands, although much simpler than collecting and rearing larvae, may provide a less predictable estimate of NPV prevalence. Mortality estimated from cadaver counts differed from mortality estimated from larval rearings by a factor ranging from approximately 2.2 to 8.8. Furthermore, mortality due to other pathogens, such as Entomophthora maimaiga, or some parasitoids, such as Parasetigena sylvestris, may not be easily distinguished from NPV-caused mortality without microscopic examination of the cadaver. In addition, the persistence or disappearance rate of cadavers under burlap bands may be influenced by factors, such as removal by scavengers, microbial degradation, or physical forces such as wind and rain, which could vary considerably among sites or years. However, the ease with which cadaver counts may be obtained may justify a sacrifice in accuracy for some applications. Therefore, this technique, as well as that of rearing larvae collected from under burlap bands deserve to be more closely examined as viable alternatives to the labor-intensive, though probably more accurate, method of rearing larvae collected from all forest strata. With additional research, it may be possible to develop a set of constants with which burlap band-based counts could be adjusted to accurately estimate NPV prevalence within gypsy moth populations.

Table 5.1. Percentage of cadavers found within each height category at site DPW, which were killed by NPV.

Height	% NPV-killed	N
Tree base	6.4	31
0.1-2m	12.2	41
2.1-4m	25.6	39
4.1-6m	33.3	42
> 6m	29.6	27

Table 5.2. Mean percentage of virus-induced mortality, among larvae reared subsequent to their collection from burlap bands which had been previously-sampled (repeat) or were sampled for the first time.

Sampling Frequency	% Mortality	(SE)	N	Number of Larvae Collected
Plot 1				
Repeat	11.5a[1]	(1.8)	27	401
First Time	9.7a	(1.2)	279	1065
Plot 5				
Repeat	8.8a	(2.1)	27	269
First Time	4.6a	(0.5)	279	984

[1]Means within a plot followed by the same letter are not significantly different ($P < 0.05$), Tukey's Studentized Range Test.

Table 5.3. Mean percentage of NPV-induced mortality among gypsy moth larvae reared on artificial diet subsequent to their collection from different forest strata and from burlap bands at three sites.

Stratum	-----DPW------- %	(SE)	N[1]	----Otis 1----- %	(SE)	N	----Otis 5----- %	(SE)	N
Litter and Understory	9.5a[2]	(2.3)	202	6.9a	(2.0)	498	4.3a	(1.2)	429
Tree Bole (exc. under burlap)	6.7a	(1.3)	363	17.3b	(3.4)	564	7.6a	(1.2)	683
Crown - Leaves	8.2a	(2.6)	215	5.7a	(2.4)	132	8.4a	(6.2)	69
- Branches	8.6a	(3.0)	101	5.4a	(1.7)	257	2.7a	(0.9)	266
Burlap	3.5a	(0.6)	1322	7.3ab	(0.9)	1451	4.6a	(0.6)	1948

[1]Total number of larvae reared.

[2]Means within a column sharing the same letter are not significantly different (P=0.05), Tukey's Studentized Range Test.

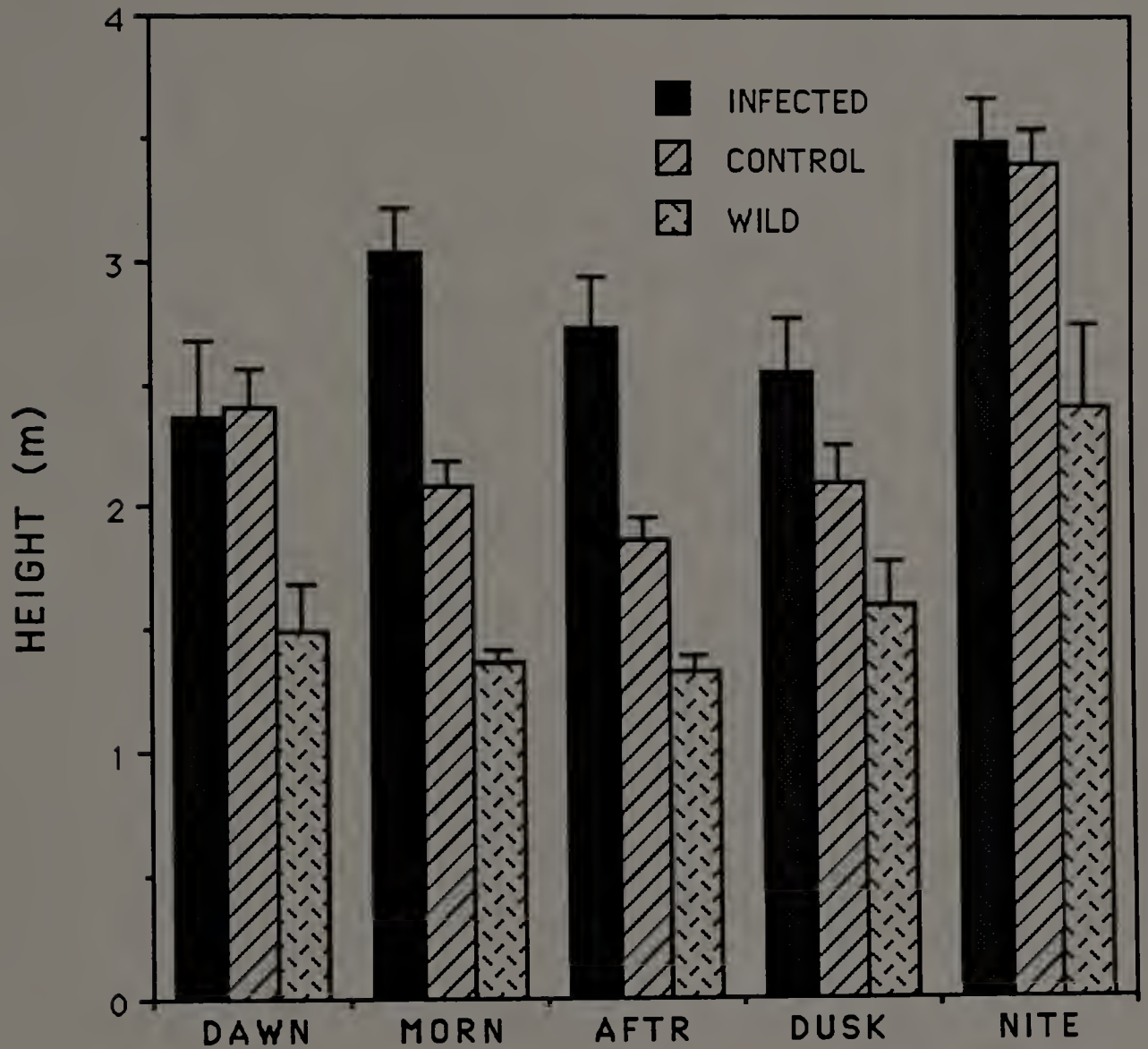


Figure 5.1. Mean height during dawn, morning, afternoon, dusk, and night hours at which live infected, control, and wild larvae were observed during the 1-10 days after their release at 1 m height onto a 6.3-m tall black oak tree.

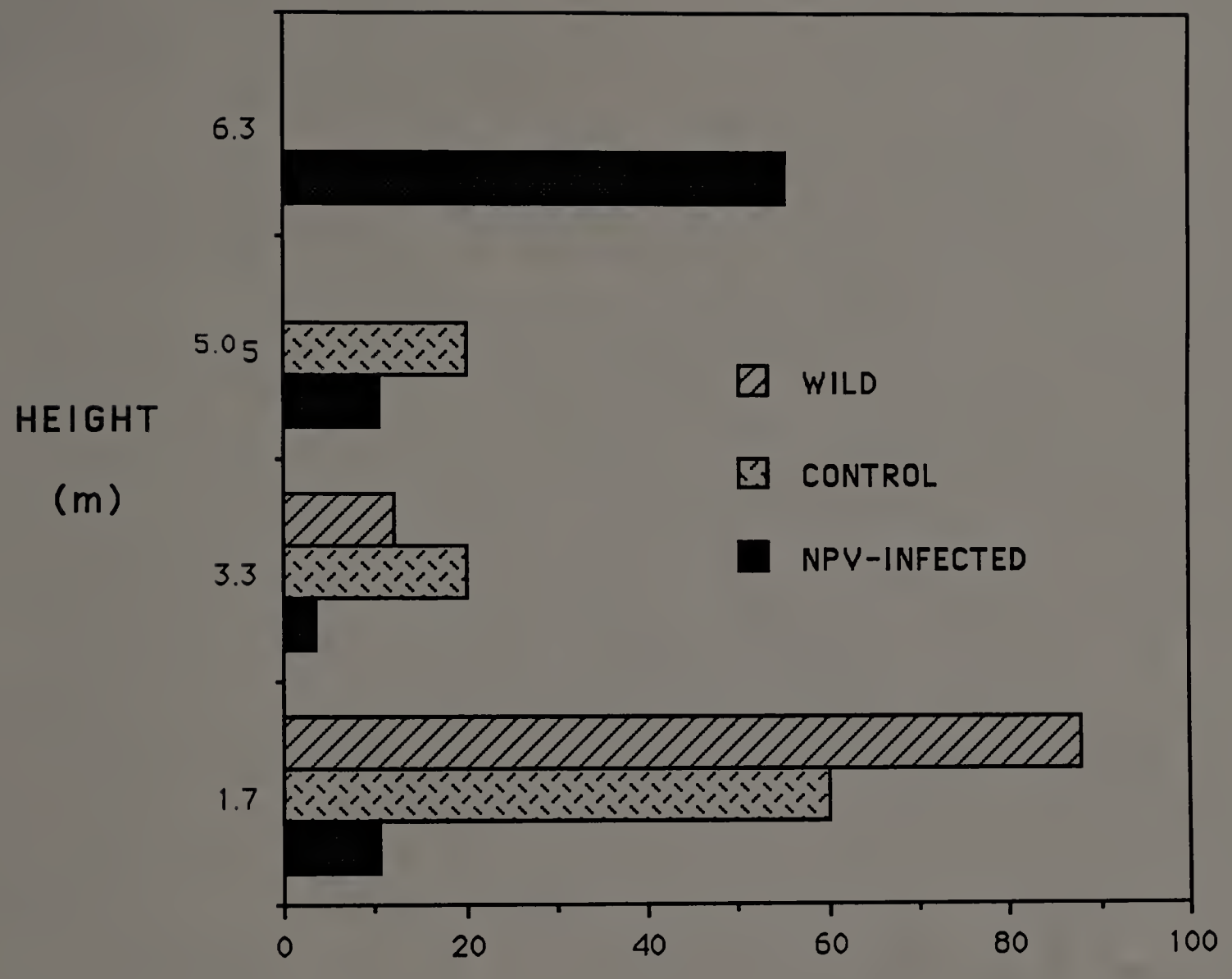
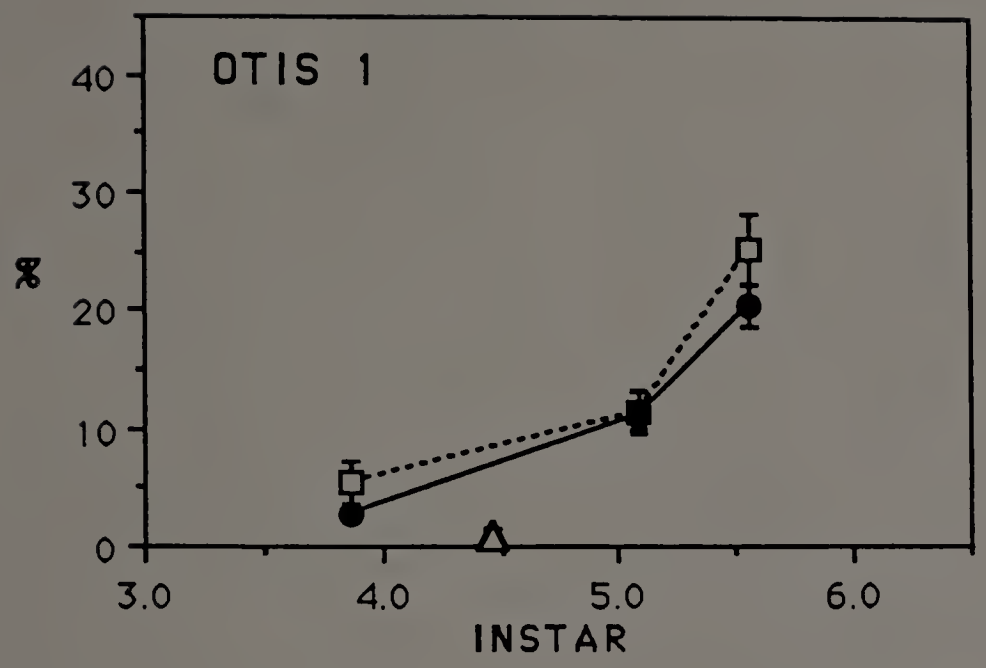
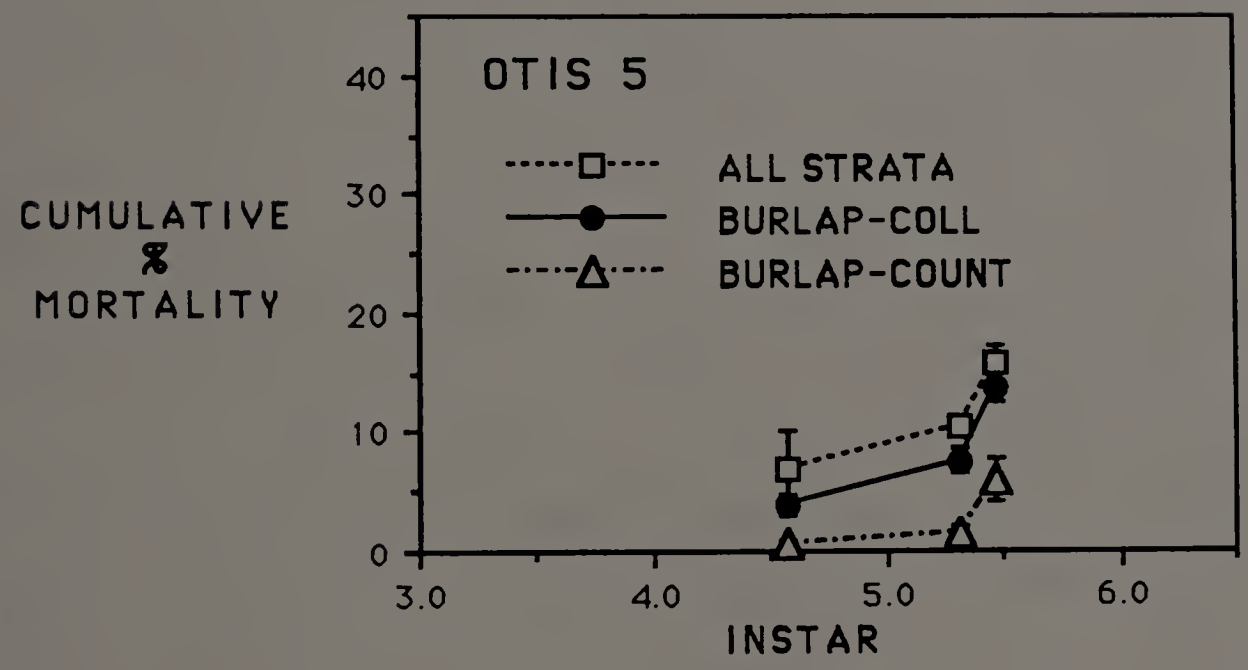
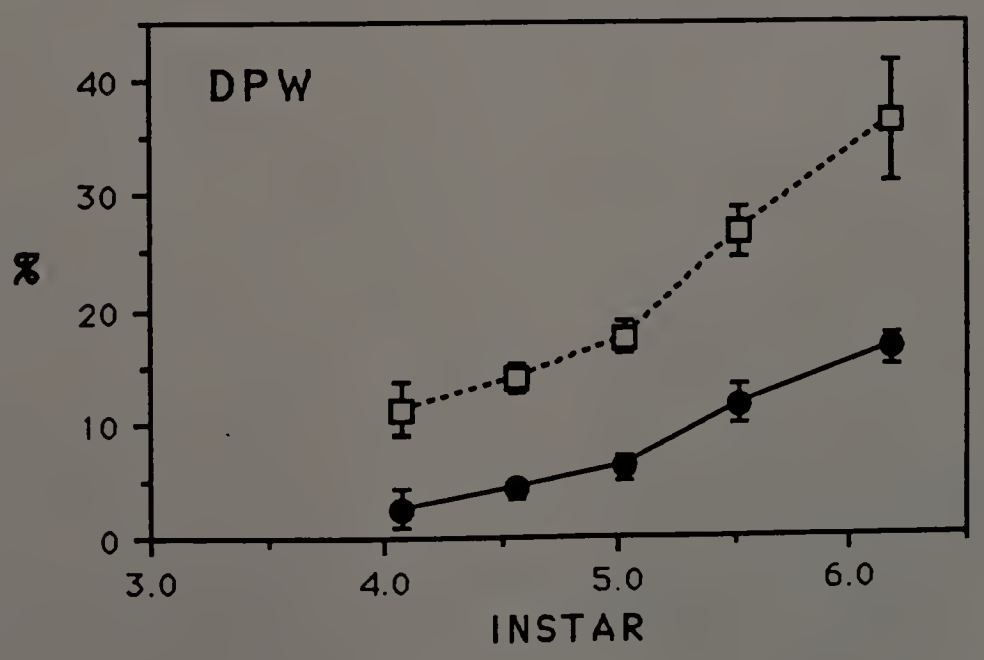


Figure 5.2. Numbers of cadavers of infected, control, and wild larvae recovered at different heights subsequent to their release onto a 6.3-m tall black oak tree.

Figure 5.3. Comparison of NPV prevalence as estimated by three methods (burlap collection, burlap counting, collection from all strata) in three sites (Otis 1, Otis 5, DPW).


OTIS 1
40
30
20
10
0
%
3.0
4.0
5.0
6.0
INSTAR


OTIS 5
ALL STRATA
BURLAP-COLL
BURLAP-COUNT
CUMULATIVE
%
MORTALITY
40
30
20
10
0
3.0
4.0
5.0
6.0
INSTAR


DPW
40
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SUMMARY

Epizootiology attempts to describe and explain the causes and patterns of disease. Complex interactions between the insect, the pathogen, and the environment influence the expression and spread of infectious disease in insect populations. An understanding of the mechanisms involved in transmission of pathogens from one host generation to the next may be necessary for successful utilization of these microbes in insect pest management programs. In addition, knowledge of the factors which affect the susceptibility of individuals and populations to infection and disease is essential to the development of insecticidal formulations of pathogens, their efficacious use, or for enhancement of naturally occurring disease.

In these studies I have demonstrated that a primary mechanism for transgenerational transmission of gypsy moth NPV is by incorporation of NPV from environmentally contaminated substrates into egg masses at the time of oviposition. Although I also found evidence that some NPV may also be deposited externally onto egg masses laid by moths which survived an epizootic (and thus were likely to be heavily contaminated themselves), most of the inoculum transferred to eggs appears to be acquired from the oviposition substrate. NPV incorporated into egg masses from the substrate maintained virulence through the winter, resulting in infection and mortality of first instars

hatching from contaminated egg masses. The dense structure of gypsy moth egg masses appears to provide the NPV protection from environmental degradation, thus, incorporation of NPV from the substrate during oviposition appears to serve as an efficient and effective means of maintaining the pathogen in the host population from one season to the next. Because NPV in egg masses is known to be a major source of inoculum for the development of epizootics, perhaps treatment of tree trunks, or burlap bands, which are also used as oviposition sites could be used as a suppression technique, instead of, or in addition to, standard spray applications targeting early instars. Treatment of egg masses or artificial contamination of substrates prior to oviposition may have an advantage over conventional larval sprays in that dispersal of infected first instars could serve to introduce NPV into newly infested sites.

I was unable to demonstrate transmission by sublethally dosed adults to offspring. In fact, even with the use of a highly sensitive DNA probe, I did not detect NPV in the tissues of adults surviving sublethal doses administered during the larval stage. These results suggest that gypsy moths do not harbor infectious NPV in the adult stage and are, therefore, unable to transmit virus to progeny, except via mechanical transfer to the egg surface by externally contaminated moths. These data cannot be considered conclusive, however. It is possible that virus can persist

in adult tissues at low levels, or in an inactive state, that eluded detection in these assays. Alternatively, perhaps the conditions necessary for sublethal adult infection and vertical transmission were not met in some unknown, but critical, way in these tests. Further research will be needed to conclusively demonstrate whether vertical transmission of NPV by infected gypsy moths is possible.

It is apparent from this study, as well as from earlier ones, that not all gypsy moth larvae that ingest NPV will become infected, nor will they all die from infection. Within a population, the susceptibility to the virus of individuals varies around some population mean. Geographically isolated populations vary widely in mean susceptibility to NPV and appear to change from one year to the next. There is evidence that population qualities associated with fluctuations in density influence a population's mean susceptibility to viral pathogens. I attempted to determine if population density or other qualities associated with fluctuating density affect gypsy moth susceptibility to NPV. While I found that mean susceptibility varied among populations and within populations across years, it did not appear to be related to population density or egg mass size. However, the prevalence of NPV in the population, as estimated from the amount of inoculum associated with egg masses, did have an effect. Populations with higher egg mass virus loads were less susceptible to NPV than were populations where NPV was

less prevalent. This suggests that exposure to NPV selects for resistance. Genetic selection for resistant strains has been shown in laboratory studies with other insects, therefore, this process might explain the results of my studies. However, this conclusion must be verified with genetic studies.

A very basic problem facing those attempting to study the ecology of an insect-microbe system, is that infection may alter the dispersion of infected individuals in a population. Thus, sampling to determine the prevalence of infection in the population, or to evaluate efficacy of augmentative releases of the pathogen in insect pest management programs may necessitate development of sampling protocol to accommodate an altered dispersion. I found that gypsy moth larvae infected with NPV climb to the uppermost parts of trees just prior to death, and that this altered behavior tends to vertically skew the dispersion of infected larvae. Sampling methods which utilize burlap bands for sampling a population tend to underestimate the prevalence of disease, because the proportion of infected individuals found under burlap bands is not representative of the infected proportion of the entire population. However, burlap band sampling may provide a rough estimate of disease prevalence.

Many ecological processes affect the expression and spread of NPV in gypsy moth populations. Although, many questions remain unanswered, we now have a better

understanding of how the virus is transmitted across generations, and some of the factors affecting host susceptibility to the pathogen. It is hoped that the knowledge gained in these studies may lead to improved utilization of NPV as a biological control agent.

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